CHARACTERIZATION OF THE PATHOGENESIS OF AMELANOSIS IN THE SMYTH LINE CHICKEN: A MODEL OF THE HUMAN AUTOIMMUNE DISEASE VITILIGO

By

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In loving rememberance of my grandmother Gertrude Chur Ho

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Autoimmune diseases result when components of normally innocuous body tissues have unexpectedly undergone changes that make them appear foreign to the immune system. The immune system recognizes aberrantly expressed self proteins as nonself and recruits an immune attack on self.

In the human autoimmune disease vitiligo, the pigment producing cells of the skin, the melanocytes, are destroyed and patients manifest irregular expanding depigmented patches of skin. An animal model for vitiligo, the Smyth line chicken, displays a spontaneous loss of feather and ocular melanocytes and the feathers progressively become whiter. Fifty percent of the birds also become blind.

By adoptive transfer of splenocytes, I demonstrated that amelanosis can be transferred and mediated by lymphocytes. This is the first direct demonstration that lymphocytes mediate the disease. Prior research by bursectomy has shown antibody mediation. A role for $\gamma\delta$ T cells was previously suggested by their expansion in number

of peripheral blood lymphocytes with age in SL chickens. Sequence analysis of the T cell receptor γδ repertoire of the peripheral blood lymphocytes indicated a polyclonal expansion, rather than monoclonal or oligoclonal, a result that might be expected if they played a direct role in antigen-driven pathogenesis. The expansion of γδ T cells may be secondary to spillover from the site of inflammation at the regenerating feather pulp. A future experiment to examine the T cells found in the developing feather may demonstrate recurrence of an oligoclonal subset of T cells. This could lead to the development of therapy aimed at inhibiting clonal T cell activation.

Southern blot analysis indicates that depigmentation does not appear to show an association with the presence of integrated endogenous viruses. The genetic heterogeneity present in SL chickens and revealed by the *ev* genotyping shows the feasibility of genetic linkage mapping to find vitiligo susceptibility loci.

There are many diseases that have autoimmune responses to what are normally innocuous everyday proteins produced in the body. Studying autoimmune vitiligo adds one more piece to the autoimmune disease puzzle. One to two out of every 100 persons suffer from vitiligo. If a common factor can be found then preventive therapies would enhance the lives of many people.

CHAPTER 1
INTRODUCTION

Autoimmunity

The normal function of the adaptive immune response to a foreign antigen is the clearance of the foreign antigen (Ag) from the body. This is mainly achieved through the B lymphocyte compartment, which develops in the marrow in mammals or the bursa in avian species, and through the T lymphocyte or thymocyte compartment that matures in the thymus. The body has learned to distinguish between self-antigens and foreign antigens during T lymphocyte ontogeny. Essentially self-tolerance is established before mature T cells leave the thymus to enter the peripheral circulation for normal surveillance. Tolerance can be induced to some foreign antigens and self-antigens in the periphery by several means. Antigen presenting cells (APCs) which include B cells and various mononuclear cells (monocytes in blood, macrophages in tissues, Langerhans cells in the skin, Kupffer cells in liver, dendritic cells in lymph nodes) process and present antigen peptides in the groove of the major histocompatibility complex (MHC) molecules.

The Ag-MHC complex engages with the T cell receptor heterodimer on the surface of a naive T cell migrating through the cortical regions in lymphoid tissue. Co-stimulation is provided by B7 on the APC engaging CD28 on the T cell. The activated T cell then remains in the lymphoid tissue, proliferates and differentiates into armed

effector T cells. T helper 1 (Th1) cells will instruct phagocytes to clear involved tissue cells containing intracellular parasites; Th2 cells will activate the corresponding B cell in the germinal centers to multiply, undergo somatic hypermutations (affinity maturation), differentiate to become plasma cells and secrete antibodies to complex the extracellular antigen or to opsonize foreign particles for recognition by phagocytes. T helper cells will also activate cytotoxic (CTL) T lymphocytes to destroy infected cells. The goal is complete and efficient clearance of the antigen from the body. Memory B and T cells are generated in preparation for a second exposure to the antigen with B cells undergoing somatic mutation adding diversity and better specificity. This is a general description of an ideally functioning immune system (reviewed in Janeway and Travers, 1997; Abbas et al., 1991).

Inappropriate responses by T cells have been suggested to initiate autoimmunity as a result of a sustained immune response against self-antigen. Inappropriate T cell help can activate a harmful antibody response against self-antigens and activate polymorphonuclear cells (PMNs) to cause tissue damage. T helper cells will recruit cytotoxic T cells. Autoimmune antibodies can bind to the target surface antigen and cause complement-mediated cytolysis of the self tissues. Autoimmune antibodies can also initiate antibody dependent cell-mediated cytotoxicity (ADCC), recruiting natural killer cells to perform cytolytic killing of the autoantigen-expressing target cell.

Autoimmune responses can be described as a loss of self-tolerance. If there is a sustained immune response that develops against the self-antigens, it becomes chronic if the initial immune effector mechanisms can not eliminate the antigen completely. The fact that the body is constantly producing and is therefore providing a constant source of

the very antigen that the immune response is against makes it nearly impossible for the vicious cycle to end. The immune response intended to protect the body from foreign intruders has in the case of an autoimmune disease launched a chronic inflammatory injury against its own tissues and may in some cases prove to be lethal.

Tolerance Mechanisms

Autoimmunity occurs as a result of a breakdown of tolerance mechanisms. Tolerance is achieved when T cells recognize antigen in the absence of co-stimulation but remain inactivated having received only one of the two required activation signals. Tolerance is established in peripheral tissues and maintained mainly by developing central tolerance of the developing thymocytes to self-antigens prior to exposure to the outside environment, and then by peripheral tolerance mechanisms. Bone marrow-derived precursor T cells undergo the process of thymocyte education by first positive selection for cells that respond to self MHC expressed on cortical epithelial cells found in the thymus. Only those that bind the self MHC molecules with some affinity but not excessively continue to the thymic medulla for negative selection. Those that do not bind or bind to self MHC too well are deleted. In negative selection, the thymic medulla presents self-antigen on self MHC molecules as expressed on bone-marrow derived dendritic cells and macrophages. Only those thymocytes that bind with some affinity but not too excessively are allowed to leave the thymus as competent mature T cells; the rest are deleted by inducing apoptosis. So potentially self-reactive T cell clones are deleted before joining the peripheral T cell repertoire. This establishes central tolerance. This has been demonstrated in mice expressing the *Mls*^{a} (minor lymphocyte stimulating) gene

product, which can interact with a high proportion of T cells expressing particular Vβ gene segments (Vβ8.1 subfamily; Vβ6). Those T cells that recognize both *Mls*[a] and the appropriate H-2 allele were eliminated during T cell development in the thymus. *Mls* is considered a superantigen (Kappler et al., 1988; MacDonald et al., 1988). Developing thymocytes that bind viral or bacterial superantigens integrated into human and mouse genomes of some strains have likewise experienced intrathymic deletion rather than anergy or peripheral suppression to provide tolerance.

Central tolerance is incomplete because not all self-antigens will be expressed in the thymus during T cell development. Therefore peripheral self-tolerance must be established. In the periphery, the mature T cell is activated by recognizing the peptide:MHC complex on a professional antigen presenting cell (APC) that must also provide costimulation of the T cell's CD28 receptor by B7 molecules on the APC. In the absence of co-stimulation, specific antigen recognition leads to anergy or deletion of the mature T cell. Those antigens expressed uniquely by peripheral organs will not normally induce clonal deletion unless transported to the thymus in sufficient amounts or brought to lymphoid tissue. This is especially the case for organ-specific intracellular self-antigens located in sequestered sites (Barker and Billingham, 1972) or self-antigens expressed below a minimal concentration level (Schild et al., 1990; Ferber et al., 1994). These are immunologically ignored because of the absence of co-stimulator activity on tissue cells. The autoreactive T cells specific for these self-antigens are usually not eliminated nor anergized unless the self-antigens become presented by professional APCs in lymphoid tissue that would break the ignorance (Aichele et al., 1996).

In the B lymphocyte repertoire, elimination of potentially self-reactive B cells occurs when immature B cells bind to multivalent membrane bound self-antigens during B cell ontogeny within the bone marrow. They are deleted by apoptosis by activation of the Fas receptor. This is unlike mature B cells, which become activated by multivalent polyclonal foreign antigens and CD4 T cell help. Self-reactive immature B cells may be rescued in the bone marrow by gene rearrangement events editing and deleting the sequence encoding for an autoreactive receptor to that of a different specificity that is tolerant (Cornall et al., 1995). B cells in the preimmune repertoire may be excluded in the competition for follicular niches in lymphoid organs if the self-reactive B cell binds soluble or low avidity autoantigens. They are not selected to proliferate and die out in the T cell zone. Such excluded B cells can be rescued if they enlist T cell help.

There are mechanisms that help mature B cells become tolerant to self-antigens in the periphery. B cells that recognize a self-antigen as they would a foreign antigen would enter the T cell zone of lymphoid tissue. However, there would not be appropriate antigen-specific armed $CD4^+$ T cells to activate the B cells. Such T cells would not have been presented the appropriate antigen from an APC and would be absent from lymphoid tissues; thus, they would not be available to provide secondary stimulation to the B cell. Those B cells with self-antigen would end up undergoing apoptosis, although death can be delayed by expression of *bcl-2*. A second mechanism is seen in naïve B cells just entering the periphery. Chronic exposure to the specific soluble autoantigen, such as soluble lysozyme, will cause them to downregulate surface IgM expression and the signaling pathways of activation in order to survive. These become anergic because they fail to generate CD28-dependent T-cell help. Normally, T cells would induce Fas-

mediated apoptotic deletion of the anergized B cell when they present autoantigen. However, in mice in which the B cells carry the Fas mutation *lpr*, the B cells are not eliminated, nor can T cells deficient in the Fas-ligand, *gld*, trigger the apoptosis of the B cells. These mice have autoimmune accumulations of lymphoid cells (Cornall et al., 1995).

B cell tolerance can be induced depending on antigen dose. High doses of antigen may overwhelm the surface immunoglobulins of B cells and induce specific unresponsiveness. This helps maintain tolerance to abundant self-proteins like plasma proteins. Very low doses of antigen in which the density of peptide:MHC complex on APCs may be too low to be recognized (that is, below the recognition threshold) by the T cells that do encounter them. Depending on the MHC genotype of an individual, some rare proteins contain peptides that may be presented at levels that are sufficient for T cell recognition but will not induce activation or tolerance. Such T cells are immunologically ignorant. They would then not be able to stimulate a B cell. T cell tolerance can be demonstrated in bone marrow chimeric animals during fetal development studies (Abbas et al., 1991). If allogeneic bone marrow is donated before the host achieves immune competence, then the developing T cell precursors would undergo central tolerance to antigens of both host and donor origin, thus tolerating self-peptides presented by both MHC genotypes.

In summary, central tolerance is established by clonal selection, strength and quality of antigen receptor signaling of the B cells, avidity of immature T cell receptors for the MHC-peptide complex, and apoptosis of deleted cells. Peripheral tolerance depends upon the need for co-stimulation by appropriate APC.

Loss of Immununological Tolerance

When the immune system is unable to remain unresponsive to self-molecules tolerance is broken and autoimmunity may result. There are organ-specific diseases and systemic autoimmune diseases. Two hypotheses suggest that autoimmunity arises due to defects in the establishment of central or peripheral tolerance, or a conventional immune response occurs against self-antigens that, under normal circumstances, did not need to establish tolerance and the tolerance became broken. As suggested by Lehmann et al., (1993) a circumvention by the display of previously cryptic host determinants to which the host never had the need to develop tolerance causes autoimmune recognition.

Mechanisms of Autoimmunity

Autoimmunity may develop against self-antigen for a number of possible reasons. As mentioned above there may be incomplete deletions of self-reactive clones due to immunologic ignorance. This ignorance (clonal escape) may be due to the differences in genetic susceptibility based on the differences in ability of different alleles of MHC molecules to bind and present autoantigens to autoreactive T cells. Thus, in healthy individuals, there are probably autoantibodies that have been characterized as consisting of unmutated germline sequences with low avidity for autoantigens, and there are antiself T cells (Schwartz, 1993).

The genetics of autoimmune diseases has been demonstrated in many ways. The HLA haplotype has often shown associations for susceptibility. For example in diabetes, people who express the MHC class II alleles HLA-DR3 or DR4, which are tightly linked to the HLA-DQ genes (the relevant disease susceptibility genes), have a noticeably higher

frequency of disease (Todd, 1995; Vyse and Todd, 1996). In HLA-DQβ1, the normal Asp-57 is substituted by an uncharged amino acid residue destabilizing the DQ molecule. Other genetic factors influence susceptibility. Identical twins have a higher frequency of having the same autoimmune disease than MHC-identical fraternal twins. The hormonal status of an individual affects disease susceptibility. Many autoimmune diseases show a strong sex bias. Diabetes in the NOD mouse is more severe and occurs at a quicker onset in the female (Wicker et al., 1986). Peak incidence of autoimmune diseases that are more common in females occurs during the child-bearing period.

If antigens are expressed selectively in a specific tissue rather than ubiquitously throughout the body, such antigens would be less likely to have induced clonal deletion of autoreactive T cells in the thymus during T cell ontogeny. Antigens of peripheral tissues especially sequestered behind anatomical barriers would not come in contact with the developing T cell repertoire. Tissue cells do not express co-stimulatory molecules. However, tissue damage may occur as a result of sustained direct attack of the cells expressing the self-antigen, from immune-complex formation, or from local inflammation. These antigens then become newly available as neoantigens in the periphery and subject to immune scrutiny by T cells that had escaped deletion. An example is seen in systemic lupus erythematosus (SLE). A broad range of autoantibodies is produced against intracellular nucleoprotein components: nucleosomes, DNA, histones, and ribosomes. Immune complexes continuously can deposit on the renal glumeruli, joints, and small arteries, and subsequently recruit macrophages to try to eliminate these immune complexes in a never ending battle (Kotzin, 1996; Schwartz, 1993).

Autommunity may be the circumvention of self-tolerance by the induction of responses to cryptic determinants to which the host was never made tolerant (reviewed by Lehmann et al., 1993; Sercarz and Datta, 1994). The responses are by members of the self-reactive repertoire that had evaded negative selection. Changes occur causing determinant spreading and the availability of neoantigens to induce activation of autoreactive T cells out of naïve T cells. Additional self-determinants previously hidden from recognition now prime other previously naïve T cells with additional specificities. Intracellular as well as extracellular proteins can be presented on class II and class I MHC (Moreno et al., 1991; Nuchern et al., 1990), leading to a wide range of newly available self-antigens. Intermolecular spreading (antigenic spread from one determinant/epitope to many in the same protein) and intermolecular spreading (antigenic spread from one protein to another) recruits more self-antigens newly available for the immune system to respond to. Endogenously produced antigens have been presented by APCs as newly recognized autoantigens on activated thyroid epithelia, hepatocytes and pancreatic cells (Dayan et al., 1991; Barnaba et al., 1989; de Berardinis et al., 1988).

In NOD mice, autoimmunity seems to start against glutamate decarboxylase (GAD) and then by determinant spreading more antigens, such as insulin, have become target antigens (Tisch and McDevitt, 1996). GAD is produced by the pancreatic islet β cells. Recent evidence is the finding of an 18 amino acid peptide showing a high sequence homology between human GAD and the Coxsackie virus P2-C protein (Kaufman et al., 1992). This is an example of molecular mimicry as a result of a misdirected immune attack.

In response to an environmental change, cytokines, such as TNF-α and IFN-γ (Pestka and Langer, 1987), may cause shifts in the peptides synthesized, and oxygen radicals have induced the heat shock protein (HSP) response. HSP have facilitated peptide binding onto the MHC (De Nagel and Pierce, 1992), and caused differences in self-peptides produced during stress. In the EAE model of T cell-mediated autoimmunity, Lehmann and colleagues (1992), showed that a single determinant of myelin basic protein (MBP), the peptide Ac1-11, was the immunodominant determinant in the primary response to MBP. Other determinants were cryptic, although available. Later in the chronically diseased mice the formerly cryptic host peptide determinants became the immunodominant primers of the second immunization. This has demonstrated diversification of the T cell repertoire due to determinant spreading.

Prior infections causing tissue damage and the inability to clear immune complexes have been suggested in the induction of autoimmune disease. There are several mechanisms that have been postulated to explain how viral involvement leads to autoimmunity (Aichele et al., 1996; Nakagawa and Harrison, 1996; Barnaba, 1996). Viruses are involved in the generation of new epitopes (neoantigens) causing a loss of tolerance (breaking of immune ignorance). Goverman and associates (1993) developed a transgenic mouse to mimic the spontaneous induction and pathology of multiple sclerosis, which expressed a TCR specific for myelin basic protein. Spontaneous EAE could not develop in a sterile environment, but it could develop easily if the mice were given pertussis virus alone or even simply housed in a nonsterile facility (Goverman et al., 1993). Anti-viral immune responses may shift and recognize shared molecular

components in self-antigens which may have altered expression in infected tissue. Thus antibodies trigger cross-reactive autoimmune reactions to shared determinants of the self-antigens (molecular mimicry) (Douvas and Sobelman, 1991). In rheumatoid arthritis, the HLA-DR β1 alleles, which contain the QKRAA amino acid sequence in the CDR3 region, have been associated with the autoimmune condition. QKRAA sequences as expressed by Epstein-Barr virus have been found in RA patients with enhanced humoral and cellular responses (La-Cava et al., 1997).

Viruses have developed means to circumvent the host. As mentioned before, viruses may act as superantigens such as the *Mls* locus for T cells with certain Vβ genes, or they may provide generalized immunosuppression, such as during HIV infection. The respiratory syncytial virus induces interferon to inhibit a proliferative response by human PBMCs (Preston et al., 1995). A T cell polyclonal activation by a bacterial superantigen could likewise overcome tolerance, as in rheumatoid arthritis or in EAE in which T cell clones expressing certain Vβ genes all become activated. The bacterial superantigen staphylococcal enterotoxin B (SEB) activates $V\beta 8^{+}$T cells that engage the amino-terminal epitope of myelin basic protein. SEB induces relapse of the paralysis in mice that are in clinical remission and triggers paralysis in mice with subclinical disease after initial immunization with the Ac1-11 epitope or after transfer of encephalitogenic T cell lines (Brocke et al., 1993). Thus incomplete deletions of self-reactive clones or aberrant stimulation or regulation of normally anergic clones later become newly elicited self-reactive clones.

Viruses can produce proteins that can regulate or counteract the antiviral responses of the host (Gooding, 1992; Marrack and Kappler, 1994). Epstein-Barr virus stimulates the conversion of uncommitted T helper cells into Th2 helper cells by the product of the *BCRF1* gene which has structural and functional homology to IL-10. This allows EBV to prevent the induction of Th1-activated inflammatory responses initiated by such cytokines as IL-1, tumor necrosis factor, and interferon-γ. The herpes simplex virus induces the infected cell to express HSV-Fc receptor, a heterodimer of glycoproteins E and I, which binds to the Fc region of the host's nonimmune IgG. This binding prevents complement-mediated lysis of infected cells by blocking access to the cell surface of antiviral antibody or effector cells (Bell et al., 1990). Cowpox virus codes for a soluble glycoprotein that has amino acid homology to that of the IL-1 receptor. This product probably competes with cell-bound IL-1 receptors for secreted IL-1, interfering with the activation of IL-1 cytokine-mediated inflammatory responses.

In persistent infections, such as in autoimmune hepatitis, the infected tissue is destroyed during long-term chronic inflammatory responses to the replicating virus itself, or is destroyed by the cytotoxic T cell response to the viral antigens presented on the target tissue. This destruction inadvertently and continuously releases large quantities of the organ's self-antigens (especially those never exposed extracellularly) which become presented by professional APCs in lymphoid tissue (Koziel et al., 1992; Cerny et al., 1994). Wounds may also disrupt tolerance by causing the release of self-antigens normally protected.

Endogenous viruses can also deregulate the expression of normal gene products. By their integration into the chromosome of the host, they cause interruptions in the normal functions of the genes and their protein products. Endogenous viruses can inactivate genes by premature termination of protein synthesis due to the addition nucleotides encoding a stop codon. Integration of endogenous viruses can create mutations that enhance transcription, and may therefore cause chronic protein expression. More about endogenous viruses will be discussed in Chapter 4.

Regulation of Autoimmune Responses

Immune regulation can either encourage the initiation of autoimmunity or act to maintain the tolerance. Cyclosporin is a potent suppressor of graft versus host disease (GVHD) and autoimmune diseases, including the suppression of amelanosis in the Smyth line chicken (Pardue et al., 1987). However, cyclosporin has also been shown to induce autoimmunity (Sorokin et al., 1986).

Adhesion molecules and cytokines can affect autoimmune processes. In the EAE model for multiple sclerosis, transforming growth factor (TGF)-β provided protection when the injection occurred for the period of 5-9 days after immunization with MBP; there was no protection if TGF-β was administered before (days 1-5) or after (days 9-11). TGF-β is immunosuppressive to the Th1-produced interferon (IFN)-γ in response to the presence of MBP (Santanbrogio et al., 1993). This Th1-mediated autoimmune disease was examined by Racke and colleagues (1995) for the role of co-stimulatory molecules. They demonstrated that *in vitro* activation of MBP-specific lymph node cells was inhibited by the combination of B7-1 and B7-2 activation. However in actively

induced disease, administration of anti-B7-1 reduced disease; anti-B7-2 exacerbated disease. In murine diabetes, intercellular adhesion molecule 1 (ICAM-1) is involved in recruiting lymphocytes to the pancreatic islet cells. Cytokines interferon-γ and tumor necrosis factor-α secreted by the islet cells could induce the ICAM-1 expression on pancreatic β cells, and immunointervention by anti-ICAM-1 and anti-LFA-1 mAbs would significantly prevent the development of diabetes (Yagi et al., 1995). Whereas the administration of cytokines promotes IDDM, the administration of mAbs against Th1-produced cytokines blocks the development of the disease (Song et al., 1996; Mossman and Coffman, 1989; Maclaren and Atkinson, 1997).

Oral tolerance has been a method of antigen-specific immunotherapy for autoimmune disease (reviewed by Hafler and Weiner, 1995; Muir et al., 1993). The use of low doses of orally administered autoantigens is suggested to utilize the secretion of downregulatory cytokines such as TGF-β and the Th2 responses of IL-4 and IL-10 to cause active suppression. High dose therapy induces anergy, the unresponsiveness of Thl function in a systemic presentation of autoantigen. Intermittent injections of the autoantigen insulin, or the B chain of insulin in incomplete Freund's adjuvant, induces an active suppressive response that induces a protective insulitis in the NOD mouse model of diabetes (Muir et al., 1995).

Autoimmune Diseases Cause by Antibodies

Autoantibodies may bind to autoantigens on the cell surfaces or extracellular matrix and initiate tissue damage similar to type II hypersensitivity. By interaction of the bound antibody with Fc receptor-bearing macrophages, there is increased clearance of

red blood cells in autoimmune hemolytic anemia. These IgG- or IgA-coated cells may fix complement to lyse these RBCs. The binding of autoantibodies to cells in tissues allows for the fixation of sub-lytic doses of the membrane attack complex of complement proteins to stimulate an inflammatory response recruiting inflammatory polymorphonuclear cells and natural killer mediated antibody-dependent cell cytotoxicity to cause tissue damage. An example of this is seen in Hashimoto's thyroiditis. Autoantibodies binding to a cell surface receptor can cause excessive activity by the receptor or inhibit its stimulation by its natural ligand. Patients become hyperthyroid in Grave's disease because antibodies to thyroid stimulating hormone prevent normal feedback to the production of thyroid hormone. Antibody response to soluble antigens produces immune complexes that are normally cleared by red blood cells, which have complement receptors, and phagocytes, which have complement and Fc receptors. Failure to clear immune complexes leads to persistent presence and deposition, especially after tissue injury continues to generate more of the antigen as in serum sickness, and chronic infections such as bacterial endocarditis, and systemic lupus erythematosus. In SLE, antibodies are formed against ubiquitously found intracellular nucleoproteins of all nucleated cells, such as DNA, RNA, and histones. Immune complexes are formed that deposit on the walls of small to medium blood vessels, especially in the renal glomeruli. These complexes attract complement and PMNs, causing more tissue damage and starting the cycle again. SLE is considered therefore a systemic rather than an organ-specific autoimmune disease.

Mothers pass on their IgG antibodies to the fetus through the placenta. Babies born to mothers with IgG-mediated autoimmune diseases may often show the symptoms

similar to that of the mothers temporarily, until the baby starts manufacturing its own antibodies. This describes one form of passive adoptive transfer of autoimmunity.

Autoimmune Diseases Caused by T Cells

Autoimmune T cells may also be directly involved in tissue destruction or in causing inflammation by activating macrophages, as well as being necessary to maintain autoantibody responses. They require the autoantigen presented on MHC with co-stimulatory ability from a professional APC and at sufficient quantities to interact in lymphoid tissue in order to initiate an autoimmune response. In insulin-dependent diabetes mellitus, the insulin-producing β cells of the pancreatic islets are selectively destroyed by $CD8^+$ T cells, which have received inappropriate activation from $CD4^+$ T cells that were activated by APCs. The specificity of the autoantigen as the target of destruction can be seen in pancreas transplants when, even though the graft is from an identical twin donor, the recipient's T cells destroy the graft.

Vitiligo in Humans

Vitiligo is an acquired melanin pigmentary disorder of the epidermis and hair follicles, manifested by expanding, irregular, depigmented lesions of the skin. Vitiligo can appear at any stage of life (LePoole et al., 1993) but half of those affected develop vitiligo before the age of 20. Vitiligo is a common disease, affecting 1-2% of the population in all racial groups worldwide. It is otherwise asymptomatic and most patients remain physically in good health. However it does predispose affected persons to sunburn skin damage and an 180-fold increased risk of melanoma (Dunston and Halder, 1990). The often severe cosmetic disfigurement has psychological effects and

current treatment modalities for vitiligo, such as phototherapy with psoralens and high intensity UV-A irradiation (PUVA), are difficult, expensive, and usually disappointing (Grimes, 1993).

Melanocyte Biology

Melanocytes, which are located in the epidermis of the skin, produce the pigment melanin, and release the melanin to keratinocytes. The biosynthesis of melanins occurs in melanocytes, and the enzyme tyrosinase catalyzes several of the initial steps of melanogenesis, which occurs within the melanosome organelle of the melanocyte (Orlow et al., 1993; Prota, 1988; Bennett, 1993). This includes the hydroxylation of tyrosine to dopa; the oxidation of dopa to dopaquinone and intermolecular circularization and oxidation of dopaquinone to dopachrome. Divergent paths then take place. In the presence of metals, dopachrome eventually becomes 5,6 dihydroxyindole, which ultimately undergoes oxidative polymerization to create eumelanin. In the presence of cysteine, the sulfur-containing phaeomelanins and trichochromes are polymerized. The black eumelanins are insoluble in all solvents and the phaeomelanins, the browns and reds, are alkali-soluble.

Two of the enzymes involved in melanin biosynthesis are characterized in studies using mutations in the mouse at the albino locus, which encodes tyrosinase, and the brown locus, which encodes tyrosinase related protein-1 (TRP-1). Trp-1 has a 43% identity to tyrosinase at the protein level. Due to two amino acid substitutions, the homozygous b/b mouse produces brown melanin which at physiologic pH, is soluble instead of the black melanin produced in the wild type which is insoluble. During

melanin synthesis, TRP-1 appears present only in unmelanized stage I and stage II melanosomes, and tyrosinase is primarily found in late stage III and IV melanosomes. Both are found in the Golgi and trans-Golgi and then enter a LAMP-1-positive (a marker for organelles of the endosomal-lysosomal lineage) organelle that is consistent with a late endosome (Orlow et al., 1993). The mature melanosomes travel along the melanocyte dendritic processes from which they are transferred to the keratinocyte for depositing.

Vitiligo Pathology

There are several theories to explain the etiology of vitiligo, including self destruction of the melanocyte, the neurogenic, the immune, and the genetic (Ortonne and Bose, 1993; Ortonne et al., 1983). Vitiligo is characterized by inherent melanocyte defects, loss of melanocytes accompanied by T cell infiltration in the affected tissue (Le Poole et al., 1993b; Hann et al., 1992; Badri et al., 1993; Erf et al., 1995b), disturbances in peripheral blood lymphocyte subpopulations (Mozzanica et al., 1990; Abdel-Nasser et al., 1992; Erf et al., 1995a), and the presence of serum autoantibodies directed against melanocyte antigens (Harning et al., 1991; Austin et al., 1992; Searle et al., 1993). Park et al. (1996) suggest that the antibodies are directed primarily against a 65 kDa antigen. Both antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated damage have been induced to cultured human melanocytes by anti-melanocyte antibodies from the sera of vitiligo patients (Norris et al., 1988). This suggests a possible role for autoantibodies in vitiligo. Two groups have demonstrated that the antibodies in the serum of vitiligo patients is against tyrosinase (MW of 70 kDa) an enzyme involved in the biosynthesis of melanin by the melanocytes (Fishman et al., 1997; Song et al., 1994).

Enzymes have been known to be autoantigens in various autoimmune diseases; GAD is a major autoantigen in the NOD mouse model of diabetes (Maclaren and Atkinson, 1997).

The melanocytes have been shown *in vivo* and *in vitro* to have intrinsic aberrant morphology, increased tyrosinase activity and increased acid phosphatase activity (Boissy et al., 1983, 1986), suggesting that an underlying melanocyte defect may predispose these cells to abnormal antigen presentation, which may be important for pathogenesis (Boissy et al., 1991). Abnormal presentation may be possible, considering the abnormal expression of class II HLA molecules by perilesional melanocytes in about 2/3 of the patients studied, as well as a six-fold increase in expression of ICAM-1 (Al-Badri et al., 1993; Ahn et al., 1994). The endoplasmic reticulum found in the melanocytes is irregularly dilated and circular rather than narrow and elongated, floccular material can be found within the cisterna (Boissy, 1991; Hafler, 1995; Im et al., 1994), and membrane bound compartments of melanosomes that contain autophagocytic activity possibly bound for lysosomal destruction have also been observed (Im et al., 1994). Yet it has not been proven that these melanocyte defects actually are toxic.

The presence of an inflammatory rim of cellular infiltrates detected in inflammatory vitiligo skin coincided with the loss of melanocytes, and infiltrating T cells in the epidermis were frequently juxtaposed to the remaining melanocytes. This rim of cellular infiltrates was in the perilesional skin in the basal layer of the epidermis and with the destruction mainly by $CD8^+$ T cells. These melanocyte abnormalities are present prior to the presence of mononuclear infiltration (Boissy et al., 1983, 1986). Keratinocytes may contribute to the HLA-DR class II presentation of melanocyte antigens following phagocytosis of melanosomes within the destroyed melanocytes (Le Poole et al., 1996).

Immunohistochemical studies have shown the actual loss of melanocytes (LePoole et al., 1993). Melanocyte loss is accompanied by epidermal and dermal lymphocyte infiltrations in the active lesions (Hann et al., 1993; Badri et al., 1993) with increases in both $CD4^+$ and $CD8^+$ T cells (Hann et al., 1993). Cellular infiltrates have IL2 and IFN-γ expressed, indicating a possible Th1 recruitment (Abdel-Nasar et al., 1994).

The Association of Vitiligo with Other Autoimmune Diseases and the Genetics of Vitiligo Susceptibility in Humans

Vitiligo is inherited as a polygenic trait and probably involves mutations in at least 3 or 4 autosomal recessive genes (Lacour and Ortonne, 1995; Bhatia et al., 1992). The risk of developing vitiligo appears to be strongly dependent on one's kinship to the proband and not dependent on gender. The relative risks, whether parent, sibling or offspring of probands, show considerable variation, pointing to a lack of involvement of a single gene with complete penetrance (Majumder et al., 1993). However, the high frequency of familial aggregation of the disease in association with other autoimmune/endocrine diseases, and the presence of organ-specific autoantibodies in the first and second degree relatives of the patients gives support to a genetic predisposition in vitiligo (Mandry et al., 1996).

Recent data suggest that human endogenous viruses may be involved in the pathogenesis of a variety of human autoimmune diseases, such as diabetes, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and inflammatory neurologic diseases (Yoon, 1990; Urnovitz and Murphy, 1996). Vitiligo may indeed be triggered by a viral infection in select patients (Grimes et al., 1996). Affected vitiligo patients can also express the hypothyroidism found in Hashimoto's thyroiditis, Grave's disease, and

alopecia universalis (loss of hair). Thus vitiligo's association with other autoimmune diseases (Elder et al., 1981; Shong and Kim, 1991; Schallreuter et al., 1994a; Nath, 1994) has categorized it within the Autoimmune Polyglandular Syndrome I diseases.

There is evidence suggesting a neuronal involvement in the disease in order to explain the segmental and symmetric distribution of depigmentation found in some patients or why there is a lack of depigmentation below the level of spinal cord injury in a patient with transverse myelitis and vitiligo. In a study of dermal nerves in vitiligo patients, Al'Abadie and colleagues (1995) concluded that cycles of initial events of vitiligo may cause axonal damage with later nerve regeneration. They suggested that the destructive mechanism of melanocytes may be triggered by the neurotransmitters released by nerve endings which are of close proximity to the melanocytes. Studies of neuropeptide and neuronal marker immunoreactivity in skin biopsies such as neuropeptide Y (NPY), support the theory that there is neuronal involvement in vitiligo and that NPY may have a role in the pathogenesis of vitiligo (Al'Abadie et al., 1994).

The C57BL/6J-*vit/vit* Mouse Model for Vitiligo

Researchers normally gravitate to the mouse in order to identify an animal model that depicts the disease condition in the human. Such exists in the C57BL/6J-*vit/vit* mouse model for vitiligo, which progressively loses much of its epidermal and follicular pigment cells during successive shedding of fur. Eyes are also affected (Lerner 1986; Lamoreux et al., 1992). This has been mapped to a recessive allele of the *microphthalmia* gene locus (mi^{vit}) (Halaban et al., 1993; Lamoroux et al., 1993). The *mi* gene has been identified as a member of a basic-helix-loop-helix zipper transcription factor family

(Hodgkinson et al., 1993) able to bind transcriptional control elements in melanocyte-specific genes. This mi^{vit} allele has a single G-to-A transition, causing an Asp222Asn substitution in the first helix domain (Steingrimsson et al., 1994). The mi^{vit} gene product of the mouse and the Mitf equivalent in the human regulate the expression of melanocyte-specific genes including TRP-1 and TRP-2 (Bertolotto et al., 1996; Yasumoto et al., 1997). However, this strain of mice does not exhibit an autoimmune component comparable to what is seen in the human, and the affected tissues fail to show a lymphocyte infiltration (Lerner et al., 1986; Boissy et al., 1987). The premature death and cytological aberrations found in this strain is considered to be the consequence of an innate cellular defect; it has been concluded that the depigmentation is the result of a genetic defect that is not initiated by a systemic or local condition (Im et al., 1994) and so it is not a suitable model for human vitiligo studies.

The Smyth Line (SL) Chicken Animal Model for Vitiligo

The Smyth line chicken represents a good animal model for the study of human vitiligo (reviewed by Smyth et al., 1981; Smyth, 1989). SL chickens are characterized by a spontaneous loss of feather and ocular melanocytes beginning around 6-8 weeks posthatch (Figure 1-1 and 1-2); thus, feathers progressively become whiter rather than maintain the original brown feather color of the parental Brown line strain (Figure 1-3).

The progenitor of the Smyth line was a spontaneous amelanotic female hatched in 1971 from the Massachusetts Brown line, and since then, a current frequency of approximately 1-2% of the Brown line spontaneously becomes amelanotic. From

Figure 1-1. A female Smyth line chicken displaying amelanosis of stage 4

Figure 1-2. A group of Smyth line chickens at various stages of amelanosis.

Figure 1-3. A typical pair of parental Brown line chickens.

outcrosses to various other chicken lines and backcrosses of the original mutant back to the Brown line, the delayed amelanosis (DAM) line, later renamed the Smyth line, was developed by selection for onset of amelanosis and severity (Smyth et al., 1981). Since the fifth generation, the Smyth line has closely resembled the parental line and three novel MHC B alleles are segregating in both lines (Erf et al., 1995a).

Pigment and melanocyte loss in SL chickens can range from partial to complete amelanosis, and about 50% of depigmented birds are blind due to melanocyte destruction in the choroid and retinal pigmented epithelium (Smyth, 1989). The magnitude of amelanosis in any Smyth line bird depends on the time frame of each feather's development when melanin synthesis and pigment deposition are destroyed. Up to 90% of a hatch will exhibit the amelanotic phenotype as reported by Smyth (Smyth, 1989) although as described in chapter 2, only about 60% are amelanotic in the University of Florida colony.

SL chickens also have an increased incidence of thyroiditis/hypothyroidism resembling human Hashimoto's thyroiditis, and a defeathering defect analogous to human alopecia (Smyth, 1989). Thus it is characterized by the same features as human vitiligo, including an association with other autoimmune diseases (Elder et al., 1981; Schallreuter et al., 1994; Shong et al., 1991). SL melanocytes have been shown *in vivo* and *in vitro* to have intrinsic aberrant morphology, increased tyrosinase activity, and increased acid phosphatase activity (Boissy et al., 1983, 1986), suggesting that, as in human vitiligo (Boissy et al., 1991), an inherent melanocyte defect may be important for pathogenesis.

Melanocyte Biology

As in the human, the melanin pigment is a product of melanosomes, the melanocyte cytoplasmic organelles that produce the pigment granules. The ocular melanocytes, as well as the choroid and anterior surface of the iris of the eye, originate from pleuripotent cells in the embryonic neural crest (Smyth et al., 1981; Smyth, 1989). The retinal pigmented epithelium and the iris are derived from the optic cup. The undifferentiated melanoblasts congregate as dermal reservoirs initially populating outside near the base of the growing feather pulp follicle (Figure 1-4). Melanoblasts migrate through the feather pulp toward the periphery of the pulp and align near the basement membrane interface of the barbed ridges and the pulp (Figure 1-5). Dendritic extensions extend from the melanocytes to the barbule cells, where melanin granules are deposited in a situation similar to the keratinocyte in the human skin. After the barbule cells receive pigment, the melanocytes retract and degenerate (Smyth, 1989; Figure 1-6).

In chickens, tyrosinase was the only known catalyst of melanogenesis (Smyth, 1989). However, more recently, tyrosinase related protein, TRP-1, but not tyrosinase, has been detected by serum autoantibodies of Smyth line chickens (Austin et al., 1995). There are five tyrosinase isozymes each with a molecular weight of approximately 66 kDa, and an additional nine other proteins that have been isolated from cultured chicken melanocytes and are assumed to be involved in melanogenesis. Dopa (dihydroxyphenylalanine) is one of the intermediate products produced by tyrosinase and can be used in tyrosinase detection (White, 1983).

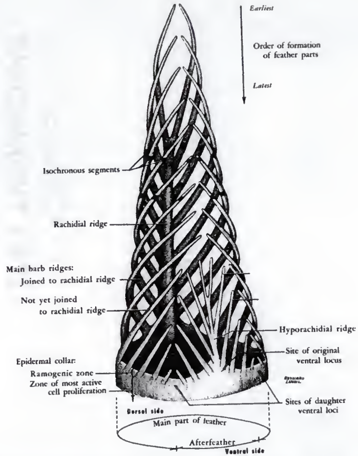

Figure 1-4. Model of a developing feather showing the arrangement of barb ridges as basilar and intermediate cells rearrange themselves into barbs. The barbs are laid out in a spiral course, extruded by the addition of cells upward away from the epidermal collar (base). They grow axially (along the shaft of) and tangentially (perpendicularly): the sum of these vectors is a spiral. The barbs are carried upward by the lengthening pulp and pushed obliquely outward. The feather (vane) will arise from the oldest or tip of the feather as the vane spreads out radially. The sheath and pulp have been removed in this depiction. Adapted from Avian Anatomy-Integument. Lucas and Stettenheim, 1972. Public domain for public use from the Agricultural Res. Service, USDA.

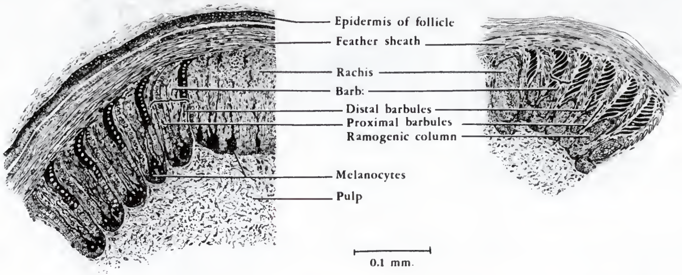

Figure 1-5. A cross section of a feather shaft and barbs perpendicular to the previous figure. The barbs radiate obliquely from the rachis (shaft) the melanocytes at the junction of the pulp and base of the feather follicle. Melanocytes have migrated from the dermis outside of the feather follicle into the pulp and congregate at the periphery of the pulp in the region of the barb cells and begin to penetrate the epidermis. They migrate in to the epidermis through the basement membrane and line up in the barbs. The melanocytes then extend dendrite processes distally outwards depositing melanin in the barbule cells. Note the outer zone of pigmented barbule cells and the inner not pigmented zone. Melanocytes are continually supplied at the base of the feather follicle; they provide pigment granules to the section of the feather as it develops and then degenerate.
Adapted from Avian Anatomy-Integument. Lucas and Stettenheim, 1972. Public domain for public use from the Agricultural Res. Service, USDA.

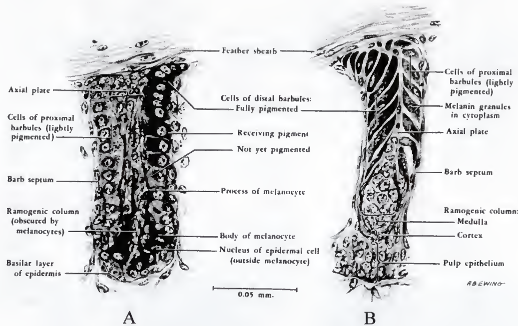

Figure 1-6. (A) A single feather barb ridge. The melanocytes extend dendritic processes distally (perpendicularly) outwards depositing melanin in the barbule cells. Note the outer zone of pigmented barbule cells and the inner not pigmented zone. Melanocytes are continually supplied at the base of the feather follicle; they provide pigment granules to the section of the feather as it develops and then degenerate. (B) In this single barb the melanocytes have degraded and disappeared after they have completed their function. Adapted from Avian Anatomy-Integument. Lucas and Stettenheim, 1972. Public domain for public use from the Agricultural Res. Service, USDA.

Amelanosis Pathology in the Smyth Chicken

As in human vitiligo patients, the Smyth line chicken melanocyte is the target of autoimmune destruction, but instead of the skin and hair follicles, the feathers, the choroid, and retinal pigmented epithelium are affected. Both autoantibodies and cell-mediated immunity may be involved in the pathogenesis of vitiligo in the Smyth line animal model. Melanocyte-specific autoAb have been detected 1-4 weeks prior to depigmentation in SL chickens, as observed in humans, and the autoAb have been shown to recognize at least three melanocyte proteins between 65 and 80 KDa, which are localized to the melanocyte cytoplasm and plasma membrane (Austin et al., 1992). Because the enzyme tyrosinase is involved in the biosynthesis of melanin, and because enzymes are known to be autoantigens in other autoimmune conditions, tyrosinase has been suggested as a possible autoantigen for vitiligo. It was recently shown that Trp-1, the most immunogenic of the tyrosinase-related proteins, is a major autoantigen recognized by serum autoAb in SL chickens (Austin et al., 1995).

The Smyth Line chicken demonstrates a functional immune system capable of providing an autoimmune response in the initiation and progression of melanocyte destruction. If the immune system is experimentally voided or suppressed then it might be expected that the amelanotic condition may be eliminated or reduced. When the B lymphocytes are essentially eliminated by neonatal bursectomy the effect is a decrease in the incidence and severity of amelanosis (Lamont and Smyth, 1981). Cyclosporin A treatment and corticosteroid-induced immunosuppression result in a decreased incidence

and severity of amelanosis in the Smyth Line chicken as long as the treatment continues. However, when the therapy is stopped the amelanosis will be as severe as littermate non-treatment controls (Boyle et al., 1987; Pardue et al., 1987).

It appears that the MHC locus influences the disease progression. Three MHC alleles have been found segregating within Smyth line chickens and sublines have been established (B^{101}, B^{102} and B^{103}). Of the three, B^{101} exhibits the earliest age of onset and the most severe phenotypes (Erf et al., 1995a).

Evidence for T cell involvement in amelanosis in the Smyth chicken is less extensive. Studies have demonstrated that cyclosporin A, a potent inhibitor of IL-1 and IL-2 release that normally stimulate PBMLs and NK cells, can measurably reduce the incidence and severity of amelanosis in SL chickens (Pardue, 1987). There is also histological evidence of an intense T cell involvement in amelanosis of the SL chicken (Erf et al., 1995b). Lymphocytic infiltration is consistently seen associated at sites of melanocyte destruction (Smyth, 1989) which have recently been shown to be primarily T cells (Erf et al., 1995b) as detected by polyclonal and monoclonal antibodies against functionally important surface T cell molecules (Cooper et al., 1991; Chen et al., 1988; Chen et al., 1989; Char et al., 1990; Lahti et al., 1988).

T cells infiltrate growing feather pulp as much as 6 weeks prior to visible signs of vitiligo. Significantly greater numbers (9-14 fold) of T cells of all three subpopulations, $\gamma\delta$ (detected by TCR1), $V\beta1^{+}$ $\alpha\beta1$ (detected by TCR2), and $V\beta2^{+}\alpha\beta2$ (detected by TCR3) are found present in cross sections of the feather pulp in SL as compared to BL prior to and throughout amelanosis. Of note are the proportions of $TCR2^{+}$ cells being

significantly higher, and of $TCR1^+$ cells being significantly lower, as compared to Light Brown Leghorn control birds (LBL, a related line with similar plumage but no incidence of vitiligo) (Erf et al., 1995b).

Initially, both the SL and control BL have a $CD4^+/CD8^+$ ratio close to 1. Prior to and early in the amelanosis, $CD4^+$ T cells are found histologically in a central, perivascular region within the confines of the feather pulp. As the disease progresses, the ratio decreases to below 0.4 (indicating mainly $CD8^+$ cytolytic cells) but then rebounds to about 0.8 late in the disease (indicating an increase in $CD4^+$ cells, probably to recruit B cells). Mainly $CD8^+$ cells remain after the melanocytes have been destroyed ($CD4^+/CD8^+$ ratio of 0.3). The shift in the CD4/CD8 ratio from below 0.4 back to 0.8 in late disease suggests the activation of T helper 2 (Th2) cells involved in a humoral response to melanocyte autoantigens released during their destruction. In the later stages of amelanosis $CD4^+$ cells become scattered throughout the pulp and surrounded melanocytes. $CD8^+$ T cells are observed throughout the pulp and are most abundant near the epithelial barb ridges and associated with melanocytes (Erf et al., 1995b). This indicates that the $CD8^+$ T cells have penetrated beyond the pulp to get to the melanin-containing barb ridges.

A working hypothesis for the pathogenesis of amelanosis found in the Smyth line chicken is that there are inherent defects in the Smyth line melanocyte that cause the cells to self-destruct. These cells may self destruct or possess a quality that predisposes them to abnormal antigen expression. Abnormal presentation by the melanocytes themselves may target them for CTL-mediated destruction. This releases the internal components of

the cell as neo antigens that the immune system responds to by the production of autoantibodies.

Genetics of vitiligo susceptibility in SL chickens

Three SL sublines have been described, and each one is homozygous for a different MHC haplotype (B^{101}, B^{102}, *and* B^{103}) based on serological typing (Erf et al., 1995a). While all three sublines are similar in incidence of vitiligo, the B^{101} SL subline has the earliest age of onset, with more severe expression of vitiligo, and a greater incidence of blindness due to retinal dystrophy as compared to the B^{102} and B^{103} sublines. Interestingly, the three SL sublines also exhibit differences in the distribution of T cell subpopulations in peripheral blood as compared to the controls, LBL and Brown line (BL, the parental line from which SL was derived, with a 2% incidence of vitiligo). B^{101} SL chickens at 40 weeks of age contained significantly fewer $CD4^+$ and $TCR2^+$ $\alpha\beta$ T cells and significantly more $TCR1^+$ $\gamma\delta$ T cells in peripheral blood lymphocytes (PBL) of 40 week-old SL chickens (Erf et al., 1995a). This increase in PBL $\gamma\delta$ T cells is detectable as early as 13-18 weeks of age (Erf and Smyth, 1996). Similar differences were found in the B^{102} subline, but not in B^{103} subline.

Chicken Immunology

Chicken Immunoglobulin genes and B cell development

Antibodies or immunoglobulins (Igs) are the antigen specific receptors produced exclusively by the B lymphocytes. They bind soluble antigens (proteins, nucleic acids, polysaccharides, lipids, and small chemicals) by recognizing conformational determinants of the antigens in their native three-dimensional form as well as determinants unmasked

by denaturation or proteolysis. During the different phases of their maturation as an adult cell, the B cell provides both cognitive and effector functions for the humoral immune response. With their membrane Ig receptors, B cells recognize and respond to specific antigens. Through their MHC class II they present processed Ag to T cells. Following antigenic stimulation they become effector cells by releasing serum Ig as plasma cells.

All progenitor B cells develop from pleuripotent stem cells that migrate from the embryonic thoracic aorta to the yolk sac, where the Ig heavy chain undergoes D-J rearrangement, and then colonize the spleen, yolk sac, and bone marrow. In these organs the cells rearrange the V_H and then the V_L genes, resulting in surface IgM expression. Between embryonic days 8 and 14 about 20,000 to 30,000 of these B cells start to accumulate in the bursa (Reynaud et al., 1987).

Mammalian B cells rely on large numbers of germline Ig gene segments and the combinatorial diversity of Ig gene rearrangement to generate a diverse Ab repertoire, which occurs continuously throughout life, in the bone marrow. In contrast, chicken Ig genes undergo rearrangement of single functional V_H and V_L gene segments within a short time period during embryogenesis. Then diversity is generated in the rearranged variable regions by somatic gene conversion using a pool of pseudogenes as sequence donors (Reynaud et al., 1987; McCormack et al., 1993).

Gene conversion provides a progressive substitution of the sequence within the functional V_L or V_H gene with sequence blocks donated or copied from the nonfunctional pseudogenes. Progressive overlapping replacement events efficiently corrects out of frame joints and expands the diversity (McCormack et al., 1993). After 6 months the

bursa involutes, and no new B cell development occurs. The B cell population is maintained by the proliferation of a post bursal population.

Chicken T cell receptor genes and T cell development

T cells recognize antigens that are linear processed fragments of foreign proteins, but only when presented to the T cell receptors (TCR) in physical association with a self MHC molecule expressed on the surfaces of syngeneic antigen presenting cells or on target cells. TCR are heterodimer plasma membrane proteins and the surfaces that bind the peptide-MHC complex are expressed as unique determinants, which differ in one clone from another, providing different antigen-MHC specificities. The particular TCR will recognize peptides associated with either class I MHC or class II molecules, which are also recognized by the CD8 or CD4 coreceptor molecules, respectively.

Both TCR $\alpha\beta$ (50kDa) and $\gamma\delta$ (40kDa) receptor molecules are disulfide-linked heterodimer glycoproteins noncovalently associated with a CD3 complex as in mammals (Sowder et al., 1998; Chen et al., 1989; Char et al., 1990). They are identified by anti-TCR antibodies: $\gamma\delta$ (TCR1) (Sowder et al., 1988), $\alpha\beta1$ (TCR2) (Cihak et al., 1988; Chen et al., 1988), and $\alpha\beta2$ (TCR3) (Chen et al., 1989; Char et al., 1990).

Chicken precursor T cells originate from pleuripotent stem cells in the embryonic thoracic aorta that then colonize the spleen, yolk sac, and bone marrow. Thymocyte progenitors enter the thymus in three waves into the thymic epithelium, which produces $\beta2$ microglobulin as a chemoattractant (Dunon et al., 1990). Each wave of progenitors will give rise to all three different forms of T cells, always in the order of $\gamma\delta$, $V\beta1^{+}\alpha\beta$, and $V\beta2^{+}\alpha\beta$ (Figure 1-7).

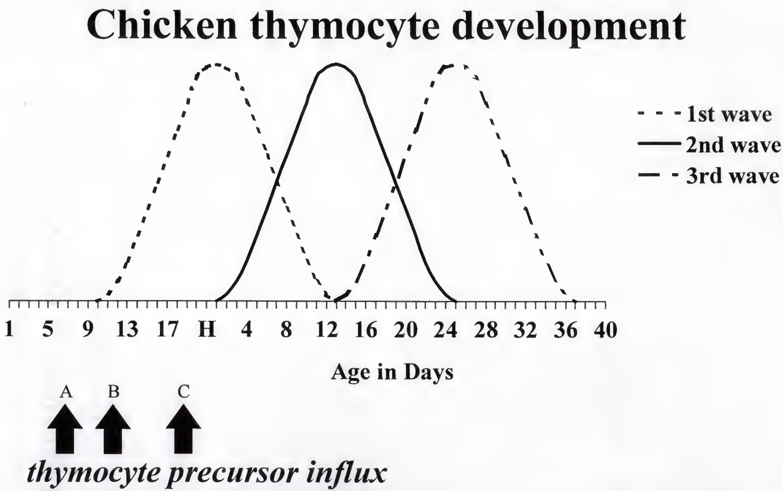

Figure 1-7. Chick thymocyte development. The arrows depict the three waves (timepoints) of entry of thymocyte stem cell precursor entry into the embryonic thymus with wave A starting at E 6.5, B at E12, and C at E18 (E=embryonic). Then the production of subsequent thymocytes leave the thymus as waves 1, 2, and 3. Each wave of production will produce T cells bearing first TCR1 receptors, then TCR2, and lastly, TCR3. The first wave is depicted here. Adapted from Cooper et al. (1991)

Chicken $\gamma\delta$ T cells (TCR1) appear first in thymocyte development, as TCR $\gamma\delta$ /CD3 cells. They enter the thymus at embryonic day 6 (E6), avoid thymic education and selection, migrate quickly through the thymus without clonal expansion, reach peak levels by E15, and exit after day E15 (Cooper et al., 1991; Dunon and Imhof, 1996). $\gamma\delta$ T cells do not express CD4 or CD8 and are not self MHC-restricted, as they do not undergo intrathymic selection (Raulet, 1989; Chen et al., 1996). In the periphery, the $\gamma\delta$ T cells reach 20-50% in the adult with a very high predominance in the intestinal epithelium and either absence or minor presence in chicken Peyer's patches and cecal tonsils (Bucy et al., 1988). They are resistant to death by receptor cross linking and apoptosis (George and Cooper, 1990) and are not subject to arrest by cyclosporin A (Bucy et al., 1990). $V\beta1^{+}\alpha\beta$ cells, in contrast, are susceptible to receptor modulation and apoptotic death (Smith et al., 1989). TCR1 cells are relatively dispersed and rarely form lymphoid nodules or aggregates even in the spleen or intestine. About two-thirds will express CD8 in the spleen and intestine (Chen et al., 1988; Bucy et al., 1988) but rarely in the circulation. $\gamma\delta$ T cells lack GVH potential and their proliferative response is relatively low (Sowder et al., 1988).

The second wave of chicken T cells enters the thymus by E12-13 and this wave contributes predominantly to the $V\beta1^{+}$ $\alpha\beta$ T cell subset. These do express initially CD4 and CD8, undergo thymic maturation and clonal selection, and are found mostly in the splenic periareriolar sheath and intestinal lamina propria with a CD4/CD8 ratio of 2/1. The last wave of thymocytes does not enter the thymus until E18 and contributes

predominantly to the $V\beta2^+$ $\alpha\beta$ T cell subset, which makes up a very small percentage of the total T cell repertoire, develop like TCR2 cells, and are found in the spleen, but are rare in the intestine. They have a 4/1 ratio of CD4/CD8. Thus the three populations are produced sequentially in agreement with their ontogeny (Coltey et al., 1989).

As in mammals, the chicken TCR genes in the chicken are structurally organized quite similarly to that of Ig chains and are evolutionarily conserved at the protein level to mammalian TCR. Key features of chicken TCR gene organization are shown in Figure 1-8. The α and γ chain variable regions are encoded by variable (V), joining (J), and constant (C) gene segments and join to form a sequence of V-J-C; the β and δ chains are encoded by V, D, J, and C gene segments, with the D, or diversity, segments between V and J in order to form a sequence of V-D-J-C. The δ chain locus is contained within the α chain locus. V, D, and J gene segments are flanked by typical recombination signal sequences (RSS) at the 3' end of V and D and the 5' end of D and J gene segments. As in the V region of the Ig molecule, there are three complementarity determining regions (CDR) and four framework regions (FR) in the V of the TCR molecule. These are involved in forming a stable three dimensional surface for binding antigen peptides presented in the major groove of the MHC molecule of an APC cell. The CDR3 is the most variable of the three CDR due to the high level of junctional diversity generated during TCR gene rearrangement.

The chicken TCR-γ locus has three $V\gamma$ families, three $J\gamma$ segments, and one $C\gamma$ segment. Eight to ten members (with high homology) are found in each $V\gamma$ family. The three $J\gamma$ segments are more closely related to each other than to any in mammalian

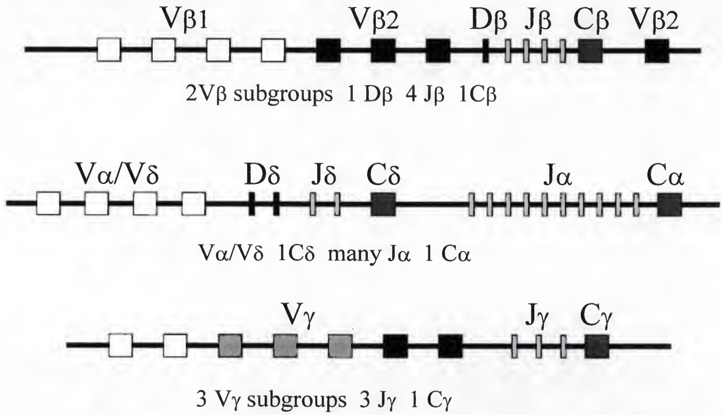

Figure 1-8. Models depicting the V, D, J, and C gene segments that comprise the genomic organization of the T cell receptors in the chicken. There are only 2 Vβ subgroups of genes, one Dβ, 4 Jβ, and one Cβ that can combine from the β chain. The δ locus is located within the α chain. There are 25 Jα but only a few get used. Compare this to only 2 Jδ . There are 3 Vγ subgroups which can join any of the 3 Jγ. (Cooper et al., 1991; Chen et al., 1988; Chen et al., 1989; Char et al., 1990; Lahti et al., 1988; Sowder et al., 1988).

species (Six et al., 1996), but most of the substitutions are in the silent second and third position in the amino acid code. TCRβ locus includes two Vβ families, one Dβ, four Jβ, and one Cβ gene segment. There are approximately 6 members of the Vβ1 family and three to five of Vβ2 gene segments. Within each Vβ family there is little difference, however, the two families bear little similarity.

So it appears that combinatorial rearrangement alone provides a somewhat limited means of generating diversity in chicken T cell receptors. No sequence modifications occur in the germline gene segments from recombination alone. In a sequence analysis of TCRβ, Cooper and colleagues realized that diversity was generated almost exclusively in the junctions, creating nontemplated N regions. Every clone was found to have a distinct sequence at these N junctions between V-D and D-J in the CDR3 (Cooper et al., 1991).

This is quite in contrast to humans and mice where there are about 50 functional V gene segments in 20-30 subfamilies of Vβ, plus two separate clusters each consisting of a single Dβ gene segment, a Jβ region (with 6-7 members each), and a single Cβ gene segment. In humans there are eight Vγ gene segments with five Jγ segments arranged in two clusters and one Cγ to yield 40 V-J pairings. In mice seven Vγ and four Jγ genes are arranged in four V-J-Cγ clusters (Arden et al., 1995a, 1995b; Janeway and Travers, 1997; Rowen et al., 1996).

T cell repertoire analysis

Despite the fact that T cell repertoires may be as large as 10^{15} specificities, it appears that most of the T cell responses studied in animal autoimmune diseases have demonstrated restricted repertoires of responsive clones, that of oligoclonal T cell

repertoires (Gold, 1994). A more refined observation is that "most pathological infiltrates are either oligoclonal in nature or display oligoclonal expansions over a polyclonal background" (Pannetier et al., 1995). This is despite the fact that there may be a difference in length in the CDR3 found in any Vβ-Jβ recombination junction of as many as 6-8 amino acids. For example, in experimental allergic encephalitis, T cells responding to the major epitope of amino acids 1-11 of myelin basic protein express Vβ 8.2 associated with either Vα2 or Vα4 (Acha-Orbea et al., 1988; Urban et al., 1988). In rheumatoid arthritis, Palliard et al. (1991) and Howell et al. (1991) suggested that a superantigen activated the preferred T cells expressing Vβ3, Vβ14, and Vβ17. Of these activated families, only T cells with specificity for synovial joint-associated antigens would then initiate autoimmune inflammatory response. These examples illustrate the limited subgroup of actual T cells clones (variants) being recruited, even though a vast number are available in the total repertoire.

If that is so, then perhaps direct targeting and functional deletion of T cells that express specific V gene products can control the autoimmunity and still maintain an otherwise intact immune system. That would be like the elimination of certain T cells that respond to the minor lymphocyte stimulating antigen (Mls) for maleness in the mouse (Scott et al., 1995). Moreover, during the course of an autoimmune disease, evidence indicates that the expressed repertoire evolves, as more antigenic determinants, previously cryptic, become available and the immune response spreads to respond to them. What may happen in the EAE system, is that the original Ac 1-11-specific T cells may upregulate self-antigen exposure in the CNS (a usually privileged site), activating a

newer set of T cells to what were until then nonsequestered cryptic MBP determinants (Lehmann et al., 1993). The reverse situation may occur in which the autoimmune response is diverse initially but honed via additional waves of recruitment, which may cause a consolidation and selection to produce a more oligoclonal T cell repertoire. So the conflicting reports between restricted or diverse repertoires may just represent different stages in the development of disease.

Other chicken models of autoimmunity

There are two other chicken animal models for autoimmune diseases in humans. The Obese Strain (OS) chicken line is characterized by iodine-induced autoimmune thyroiditis, and is recognized as a model for the organ-specific disease Hashimoto's thyroiditis. Reducing thyroidal iodine by antithyroid drugs can prevent the thyroiditis. However, therapy must be administered at the embryonic stage (Bagchi et al., 1995); otherwise, the thyroiditis becomes severe by 5 weeks of age. Autoreactive B and T cells can be seen in the thyroid by 2 weeks post hatch (Wick et al., 1970). Furthermore, adoptive transfer of splenocytes from affected OS chickens to the Cornell strain (CS), a related strain that develops a mild late onset disease, causes the development of thyroiditis when the hosts were supplemented with iodine (Brown et al., 1991). It has been recently demonstrated that the T cells expressing Vβ1 genes are the main T cells infiltrating the OS strain thyroids (Cihak et al., 1995).

The University of California at Davis (UCD) lines 200 and 206 chickens develop a hereditary scleroderma-like connective tissue disease. It develops early in life, as early as 7 days post hatch, presenting initially as swelling, erythema, and necrosis of the comb,

digits, and skin (Haynes and Gershwin, 1983). Survivors develop a severe lymphocytic infiltrate of the comb, skin, digits, and viscera. They develop an excessive buildup of collagen, resulting in fibrosis of the dermis and as vascular occlusions of internal organs such as the esophagus, small intestine, lungs, kidneys, heart, and testes. T helper and T cytotoxic cells are present with a CD4:CD8 ratio of 1.44:1 by week four (van der Water et al., 1989). The infiltrates also contained distinct groups of B cells as the disease progressed. These infiltrates secrete IgM, fibroblast-activating cytokines (Duncan et al., 1995), antinuclear antibodies (including antibodies to ssDNA), and anticytoplasmic antibodies that recognize an avian-specific set of antigenic determinants (Haynes and Gershwin, 1983). As in humans, fibroblast activation is suggested to contribute to fibrosis (Duncan et al., 1992). A defect in the T cells' response to a panel of T cell mitogens such as concanavalin A or pokeweed mitogen indicates abnormalities in T cell stimulation as seen in decreased calcium influx and proliferation (Wilson et al., 1992).

Limitations in the use of the chicken animal model

No animal can represent perfectly what is found in a human. Small mammals have become the more popular study models and often they can depict the human phenomenon reasonably well. In the case of vitiligo, the chicken presents a closer animal model due to the shared features suggesting an autoimmune component to the disease. However, the chicken is not well regarded as a relevant animal model for medical research, certainly not as extensively as the laboratory mouse. With a much more limited pool of investigators and experience, there is not an extensive network of shared technical protocols that have been developed or people aware of the chicken system to

begin trouble shooting this untapped resource. It takes time and a collective effort to try new ideas in the chicken just as it was for pioneers with the small mammals. Beyond not knowing if a protocol may work for a specific strain is whether the protocol already established in the mammal can be adapted to the chicken at all.

It takes 5-6 months for chickens to become sexually mature so creating congenic chickens with 12 or so crosses would require several years. Slow reproduction is a problem with animals larger than mice. Larger animals are more costly to feed, house, and have enough space for. Because the chicken is not used extensively, researchers do not attempt new technology with them and the biotechnology industry does not find the need to develop useful tools. Transgenic chicken embryos are created with the assistance of infections by variants of the Rous Sarcoma Virus. Hybridomas have been developed only in the last few years.

The genome of the chicken is just beginning to get serious attention and now only in the past 2 years has linkage mapping of the genome with readily available microsatellite markers is being started. Unlike the mouse, chickens have not been characterized genetically into well-defined lines guaranteeing the purity of a line. There is no equivalent for chicken of a library such as that of Jackson Laboratories that allows a scientist to buy a mouse, C57BL/6, or the NZW for its specific genetic features. The library of described avian cell differentiation antigens and known avian cytokines and lymphokines is also less extensive than that of the mouse and human. This limits the extent of some types of avian research, such as the cytokine profile expressed by cells in response to inflammation of the thyroid in the Obese Strain chicken.

A conclusion that can be made about using the chicken to study diseases in the human is that it will probably not be as readily appreciated as the laboratory mouse. It is more expensive in terms of reproduction time and cost to maintain. One really needs to be both a poultry and medical scientist. The typical medical scientist is not aware of the extensive network, knowledge, and experience that is necessary to study live chickens.

On the other hand, the chicken is the best studied vertebrate for embryogenesis and development. The egg provides a most convenient source of embryos; the shell can simply be opened to expose the living animal. It was through studying the chicken, that the concept separating the B cells as bursa-derived (bone-marrow-derived for mammals) from the T cells, as thymus derived, was clarified. Several monoclonal antibodies including those that distinguish several subsets of T cells and B cells are available and marketed for cell separations and immunohistochemistry. At this moment, the Smyth line chicken does represent the closest model providing the best opportunity to unveil some of the unknown pathology of human vitiligo.

Rationale for this study

Vitiligo is considered an autoimmune disease. Vitiligo is not considered life threatening such as the physiological destruction of the insulin-producing cells in diabetes; nevertheless, for the 1-2% of the population with vitiligo there is a significant increased risk of skin cancer. The patient's well being and self esteem are compromised and these may cause distress because there is little the patient can do to hide the condition. The current means to treat vitiligo is to have the patient undergo PUVA

therapy to attempt to stimulate new melanization However the treatments are harsh, prolonged and the success rate is poor.

Often the presence of one autoimmune condition can be found to occur along with other preexisting autoimmune conditions and this is true for vitiligo. For example, vitiligo has been associated with Autoimmune Polyglandular Syndrome type 1 and vitiligo patients have increased risk for other autoimmune diseases.

Animal models have proven to be very useful in studying the pathogenesis of autoimmune diseases. In the study of diabetes mellitus, the target of autoimmune destruction is the β islet cells of the pancreas. The NOD mouse has been a very useful animal model to study the human disease. The insulitis has an infiltration mainly of $CD8^+$ T cells but also of $CD4^+$ T cells to a lesser degree. The disease has been proven by many labs to be adoptively transferred using T lymphocytes. Immunotherapy targeted at the T cell has been used inhibit the progression of the disease. Autoantibodies against the islet cells are characteristic of diabetes. They proceed and are detectable before the onset of the disease. GAD has been identified as a major autoantigen; both forms of which have homology with a peptide from Coxsackie virus. Islet cell antigen (ICA) has more recently been identified as a autoantigen in diabetes. Yet, the role of the autoantibodies in causing the destruction is not clear.

The Smyth line chicken is the best available animal model for the study of human vitiligo. The melanocytes of the regenerating feather compare to the melanocytes of human epidermis and hair follicles. The depigmentation develops in patches that are irregular and expand as the process proceeds. The disease is otherwise asymptomatic in

both species, except in nearly 50% of the affected that become blind as well. There are similar inherent defects within the chicken melanocyte. An intense lymphocytic infiltrate with increased numbers of CD8 and CD4 T cells is associated in the chicken. Autoantibodies are detectable before the onset of amelanosis. A putative autoantigen, tyrosinase-related protein, has been detected and is related tyrosinase which is an enzyme involved in melanogenesis (Austin and Boissy, 1995).

Thus in this study of vitiligo, experiments were designed to ask some of the same questions as in other animal models of autoimmunity. Adoptive transfers of lymphocytes from affected SL chickens into non-affected BL chickens tested the hypothesis that autoimmune lymphocytes can induce the destruction of the feather melanocytes and cause the depigmentation. Likewise, the hypothesis that sera containing autoantibodies might induce disease if transferred to an unaffected host was also tested.

Since T cells have been shown in diabetes to cause the disease, the question of which subsets of T cells might be the key members involved would help define the pathology. In the peripheral blood of Smyth line chickens, the $\gamma\delta$ T cells increase in proportion during the course of the amelanosis and with age. Therefore, a repertoire analysis of the $\gamma\delta$ T cells in the peripheral blood was examined.

Since animal models allow one to manipulate the genotype of the animals to determine genetic causes of diseases, preliminary genetic studies of the SL chickens were performed. If certain patterns of inheritance are found that would correlate with the presence, absence, onset, or severity of the disease then this would help in the understanding and predictability of the disease. Endogenous viruses are genes that

encode components of retroviruses that have become integrated in the genomes of all species of vertebrate animals. They are stable and inherited in Mendelian fashion. The random integrations provide unique genetic markers that can be used to follow inheritance and examine for correlations with phenotype.

It is a goal of using animal models to provide a means to gain understanding of a human disease. This study of the amelanosis in the Smyth line chicken is being pursued to understand vitiligo in humans.

CHAPTER 2

ADOPTIVE TRANSFER OF AMELANOSIS IN THE SMYTH LINE CHICKEN

Introduction

This aim is designed to determine the role and clarify the contribution of humoral and cellular immunity in the pathogenesis of amelanosis in the Smyth line chicken.

In order to study and characterize the immune response, it is often an advantage to study the intact organism. To make the study manageable, experimental animals have been manipulated by various means to help study immune functions. The laboratory mouse for example, has been inbred so that the immune responses based on the MHC haplotype have been characterized and documented to minimize 'masking' of the effect of a locus or genetic region; a large collection of MHC-specific strains is available through Jackson Laboratories. This allows researchers to choose mouse strains to conduct investigations and make variants such as the NOD mouse. The variants have been developed through altering the genome either by inserting new genes to create transgenic animals, or by targeted disruption of genes by gene knockout through means of homologous recombination.

Adoptive transfer of cells or antibodies is a classical experimental approach to demonstrate immune function. The transfer of serum (the fluid phase of blood containing specific antibodies against an immunizing antigen) from an immunized individual (donor) into a naïve individual (host or recipient) can confer immunity if antibodies

mediate that condition. This is passive immunization, dependent upon the antibodies generated originally by the donor by active immunization or infection. Adoptive transfer of lymphoid cells from the immune donor can provide cell-mediated immunity in a host. Transfer of cells must be done between donors and recipients genetically matched at the major histocompatibility complex (MHC) loci so that the donor cells are not rejected by the recipient and do not attack the recipient's tissues (graft versus host disease). Incompatibility may also occur despite the donor and host being identical at the MHC locus, due to differences in the minor histocompatibility antigens (Scott et al., 1995), such as the male specific H-Y antigen.

Immunosuppression of the host animal is often utilized to facilitate adoptive transfer studies because syngeneic matches in the MHC are rare in outbred populations. This pretreatment also provides a void in the immune function in the recipient host providing space for the restoration of immune function by the adoptively transferred cells (Toivanen et al., 1975). This allows the effect of the transferred donor cells to be studied in the absence of host lymphoid cells. One method of immunosuppression is by the use of ionizing radiation from X-rays or γ-rays to kill off rapidly dividing lymphoid cells at doses that spare the other tissues of the body. Other means of cell depletion include neonatal thymectomy, cyclophosphamide (which acts primarily by eliminating B cells and suppressor T cells) (Toivanen et al., 1975; Harada and Makino, 1984), splenectomy, and antilymphocyte antibodies generated in another species of animals.

Adoptive transfer studies have been performed to study the functions of chicken lymphocyte subsets. Toivanen et al. (1975) compared the transplantation of lymphoid

cells (bursa, spleen, or bone marrow) into 4.5 week old immunodeficient chicks. Using donated bursal cells of 3 day old, 4.5 week old, or 10 week old donors, and pretreatment with cyclophosphamide (Cy), even large numbers of donated bursal cells would not bring about a long term restoration of antibody formation. Pretreatment by X-irradiation (750 rads) on the day before transplantation with 10 week old donated cells from spleen (as well as marrow, thymus, or bursa) allowed higher survival rates and body weight gains suggesting that restoration of T cell functions, but not B cell functions, was achieved. Toivanen and colleagues concluded from these studies that T cell function is more crucial to survival than is humoral immunity. A dose of 750 rads for 4.5 week old chickens was shown to be effective in allowing reconstitution of the T cell compartment, but only short term reconstitution of B cells.

Lehtonen and co-workers (1990) determined that Cy treatment destroys proliferating B cells in the bursa, and allows donor B cell reconstitution in 4 day old hosts for at least 10 weeks if 4 day old donor bursa cells were used. Irradiated with 750 rads, 4 day old hosts could again be reconstituted with T cells but not with B cells. The B cell compartment was not restored. Based mainly on these reports, our experiments utilized irradiation treatment for the adoptive cell transfer of lymphocytes from amelanotic SL chickens into BL hosts, in order to determine whether the autoimmune disease could be transferred by lymphocytes.

The transfer of autoimmune disease to host animals has been previously demonstrated by the adoptive transfer of lymphocytes in other animal models of autoimmunity. Experimental allergic encephalomyelitis (EAE) is a disease produced by injecting animals with homogenized spinal cord, myelin basic protein (MBP), and it

resembles the demyelinating disease similar to multiple sclerosis. It has been transferred using MBP-reactive T cells from the spleen or lymph node cells from a MBP-immunized donor to naïve syngeneic hosts in mice and rats (Panitch and McFarlin, 1977; van der Veen et al., 1989). In EAE, it appears that $CD4^+$ T cells expressing a restricted, limited TCR repertoire are responsible. In humans, however, the TCR repertoire may be more diverse, with greater heterogeneity of MBP-specific T cells associated with a greater severity of disease (Richert et al., 1995; Utz and McFarland, 1994; Utz et al., 1994). Systemic lupus erythematosus was transferred to SCID mice when human PBMC were injected into SCID mice, and the SCID serum was shown to carry the human autoantibodies for up to 22 weeks (Ashany et al., 1992).

In the non-obese diabetic (NOD) mouse, a model for human insulin-dependent diabetes mellitus, several laboratories have demonstrated the transfer of insulitis and diabetes into irradiated hosts. Normally, signs of initial insulitis begin to appear by the sixth week. By 30 weeks of age, spontaneous diabetes develops in about 95% of the mice with a mononuclear cellular infiltrate within the pancreatic islets (Wicker et al., 1986). Wicker and coworkers induced diabetes within 3 weeks in greater than 95% of the hosts when the hosts were older than six weeks of age and by using unfractionated splenocytes from overtly diabetic NOD donor mice older than 16 weeks old (Wicker et al., 1986). They refined these studies further by achieving successful adoptive transfers using splenic T cells using the $CD4^+$ or $CD8^+$ T cell subsets (Miller et al., 1987). Meanwhile, Hanafusa and colleagues (1988) induced insulitis in T cell-depleted NOD mice reconstituted with the same two T cell subsets. Subpopulations of spleen and lymph node cells transferred diabetes to syngeneic neonates and demonstrated an age

and cell dose-dependent susceptibility range (Bendelac et al., 1987). LaFace and Peck (1989) transferred diabetes in non-susceptible C57BL/6 or B10.BR/cd mice, and Serreze and co-workers (1988) did the same in NOD SCID hosts. T cells have been generally recognized as being mediators of autimmunity against the pancreatic β cells in diabetes.

Autoantibodies can also be transferred from autoantigen-immunized donors to induce the autoimmune disease in hosts. Autoantibodies to the thyroid stimulating hormone receptor from mothers with Grave's disease frequently produce thyroid activation when serum is transferred into the fetus. Because IgG can cross the placenta, infants of affected mothers can be born with hyperthyroidism (Gossage and Munro, 1985; Becks and Burrows, 1991). Thyroiditis enduring for up to 40 days has been induced by the transfer of antiserum to susceptible strains of mice (Tomazic and Rose, 1975). Likewise in the Obese Strain chicken, the chicken model for thyroiditis, repeated injections of high titer antiserum for 4 weeks induced thyroiditis (Jaroszewski et al., 1988). In EAE, serum transfer in a rabbit model induced severe autoimmune thyroiditis (Inoue et al., 1993). Autoimmune cataract formation has been created experimentally in eyes of mice by means of serum and monoclonal antibody transfers from donors which had received injections of emulsified beta-crystallins (Singh et al., 1995).

A current hypothesis of the pathogenesis in the Smyth Line chicken suggests that there are inherent defects in the melanocytes, predisposing SL melanocytes to abnormal antigen presentation (Smyth, 1989; Austin and Boissy, 1995; Sreekumar et al., 1996). Cells and antibodies in the SL chicken become sensitized to melanocyte antigens. Presumably auto-reactive T cells associated with the melanocytes can be seen infiltrating

the feather barb ridges in tissue sections stained with monoclonal antibodies specific for T cell markers (Erf et al., 1995b). Austin and colleagues reported the detection of melanocyte-specific antigens between 65 and 80 kDa in the Smyth line chicken (Austin et al., 1992). More recently, Austin and Boissy (1995) reported that these same autoantibodies are recognizing the chicken homologue of mammalian tyrosinase-related protein-1 (TRP-1).

Consideration must be given to the fact that during a long term condition as in autoimmunity changes will occur in the autoimmune repertoire during the course of a disease. Autoimmunity may represent not only the breakdown of self-tolerance, but the display of new cryptic self-determinants to which the host was not originally tolerant (Lehmann et al.; 1992 and 1993; Sercarz and Datta, 1994). The changes in the antigenic determinants that are involved in this amelanosis may therefore be reflected in a changing autoimmune T cell and B cell repertoire. The design of adoptive transfer experiments, might, therefore, take into account these possible shifts in antigenicity, and utilize donor cells from donors of different ages.

The parental Massachusetts Brown Line chickens (BL) from which the Smyth line was derived also exhibit the amelanosis of the feather and eyes but at an incidence of only 1 to 2 percent (Erf et al., 1995a) as compared to the 90% incidence reported for SL (Smyth, 1989; Smyth et al., 1981). One potential explanation for this low incidence of amelanosis might be that the melanocytes of some BL chickens display the same defects in antigen presentation as hypothesized for the SL. The adoptive transfer of autoreactive lymphocytes from amelanotic SL chickens may result in the same autoimmune pathogenesis. Alternatively, the melanocyte defect may not be required, and the simple

presence of anti-melanocyte autoreactive lymphocytes, or autoantibodies, may be sufficient to cause amelanosis in BL chickens receiving SL lymphocytes or serum autoantibodies by adoptive transfer. The variables tested included the host age, donor age, use of irradiation, and number of injections of donor lymphocytes. We also performed one experiment involving the transfer of SL serum autoantibody into BL hosts. In this study, we report the first transfer of autoimmune amelanosis with splenic cells from Smyth Line chickens in Brown Line chickens.

Materials and Methods

Animals

Fertile SL eggs from the B^{101} major histocompatibility complex (MHC)-defined subline, which has the earliest age of onset and the most severe phenotype of the three SL MHC-defined sublines (Erf et al., 1995a), and from the B^{101} MHC-matched BL were generously provided by Dr. J. Robert Smyth, Jr. (University of Massachusetts, Amherst). Chickens were hatched and housed at the University of Florida Poultry Unit, and were individually identified with leg band or wing tag numbers. The degree of pigment loss (amelanosis) by SL chickens was classified according to Erf et al. (1995b): (1) normal, no apparent amelanosis; (2) mixed amelanosis, with both normal and <20% amelanotic feather tissue; (3) mixed amelanosis, with normal and 20-60% amelanotic feather tissue; (4) mixed amelanosis, with normal and >60% amelanotic feather tissue; and (5) complete amelanosis, all developing feathering tissue is amelanotic. SL chickens with stage 1 or no amelanosis are also referred to as nonprogressors, and SL chickens with any apparent

amelanosis (stages 2-5) are referred to as progressors. It should be noted that the phenotypes reported represent the maximum amelanosis stage reached and were stable.

The SL donors were hatched and raised before hatching the BL hosts so that there were visible amelanotic donors by the time they were 8-12 weeks old in time to donate their cells to 3 week old BL hosts.

Sex Determination by PCR

The sex of the donors and the recipients was determined when the hosts were 3 weeks old because the hosts at this age do not have definitive secondary sexual features for reliable identification. SL females display the earlier onset and more severe phenotype than SL males and would appear to have the best potential of passing autoreactive T cells. The ideal transfer would be from a SL female to a BL female. Attempts were made in some experiments to avoid female to male cell transfers in case of possible incompatibility at the minor H loci. In birds, the females are the heterogametic sex, bearing the W and Z chromosomes whereas males are ZZ. The gender was determined by PCR using primers, Chi I and Chi III, derived from the sequence of the chicken W chromosome-specific Xho repeat fragment described by Kodama et al. (1987) and kindly provided by Dr. Siwo R. de Kloet (Florida State University, Tallahassee). The DNA template for the PCR was obtained from a drop of blood obtained by pricking the brachial wing vein and absorption onto Isocode Stix, PCR template preparation dipsticks (Schleicher and Schuell Inc., Keene, NH). Genomic DNA was eluted and PCR amplified as directed by the manufacturer. A 316 bp product was resolved on a 1% agarose gel.

Immunosuppression of the Host Animals

Some host BL chicks were immunosuppressed by sublethal irradiation one day before the cell transfers. γ irradiation was performed using a ^{137}Cs source at the U.F. Health Center Animal Resources Facility. Dosages used were either 750 rads (Toivanen et al., 1975, as used on 4 day old chicks) to 850 rads (Dr. Bruce Glick, personal communication) or none at all.

Preparation of the SL Donor Cells and Cell Injections

The donor SL cells were obtained from SL chickens undergoing active amelanosis at the time of sacrifice (8 to 20 weeks of age). The spleens were removed, made into cell suspensions in PBS on ice by teasing the organ and by dounce homogenization, and the lymphocytes were separated from the red blood cells by density centrifugation over Ficoll-Hypaque (Pharmacia). The splenic lymphocytes were resuspended in PBS at a density of $5x10^7$ to $1x10^9$ cells per ml, and a maximum of 1-2 ml of cell suspension was then injected intravenously into the right jugular vein with the balance into the wing brachial vein. The recipient animals were then monitored in normal housing conditions (not pathogen-free) for at least 20 weeks of age to allow the development of the amelanotic phenotype during the typical time frame as would be found in a SL chicken.

Smyth Line Serum Collection and Preparation

Serum was collected from Smyth Line chickens that displayed obvious amelanosis (at least level 3) at the time of collection. A range of 20 to 60 ml of blood per bird was collected into heparinized Vacutainer tubes from the jugular and/or brachial vein of the wing. Blood was centrifuged at 1500 rpm to collect the serum, which was

then stored at -80°C. The sera was then pooled and the gamma globulin fraction was obtained by two sequential precipitations with 33% and then 28% saturated ammonium sulfate. The precipitates were dissolved in a minimum volume of cold phosphate buffered saline (PBS), dialyzed 2-3 days against 4°C PBS after each precipitation, and then filter-sterilized using a .45μ micropore filter (Nalgene). Aliquots of the gamma globulin fractions were then introduced by injection in the jugular vein (1-2 ml) with an additional volume (2-3) introduced intraperitoneally beneath the breastplate.

Cell Lines

Chicken melanocyte cultures were obtained from chicken embryonic neural crest tissue by the method of Boissy and Hallaban (1985) in the laboratory of Gisela Erf and obtained as a gift. Cultures were grown in Ham's F10 media (Sigma) supplemented with 10% FBS, 5% Nuserum (Collaborative Biomedical Products), 200 mM L-glutamine/penicillin-streptomycin (Sigma), 0.5 mg/ml cholera toxin (Sigma), and 1mM phorbol myristic acid.

Immunoblotting

Semiconfluent melanocytes were harvested from flasks, rinsed twice in PBS and solubilized in 10 mmol/L Tris buffer (pH 8), with 1 mmol/l phenylmethysulfonyl fluoride, 5% 2-mercaptoethanol, .02 mM/l of each antipain, aprotinin, chymostatin, leupeptin, and pepstatin A, with 1% sodium dodecyl sulfate (SDS) for 5 minutes at 95-100° C, and sheared with 21 GA needle. The lysates were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reducing gels and electroblotted onto Immobilon-P polyvinylidene fluoride (PVDF) membrane. A Bio-Rad low range

molecular weight marker lane was used (Bio-Rad Laboratories, California). The blotted proteins were blocked in 5% Carnation non-fat dry milk with 20 mM Tris pH 7.5, 137 mM NaCl, and 0.1% Tween 20 (TBS-T). The blots were cut in individual strips and each strip was individually incubated with different sources, primary antibody (either direct serum cleared as described above or the gamma globulin fraction) for 2 hours at room temperature in TBS-T with 5% milk, washed 5 times for 2 minutes, and 2 times for 5 minutes and then incubated in goat anti-chicken horse radish peroxidase-conjugated secondary antibody (Southern Biotechnologies, 1:1500) for 2 hours. The proteins were detected by ECL chemiluminescence (Amersham). The blots were analyzed by densitometry and Hoefer Image Master software.

Histology

Regenerating feathers of SL and control BL birds were generated by gentle plucking of growing feathers and collecting of the young feathers bimonthly. The feathers were brought to the University of Florida Diagnostic Research Laboratory for cryosectioning. Mouse monoclonal antibodies to chicken TCR1, TCR2, TCR3, CD4, CD8, CT3 were a gift of Dr. Chen-lo Chen and described by Cooper et. al., 1991). Methyl green was used as the counterstain.

Results

Observations of the UF Colony of Smyth Line Chickens

Observations have been compiled from raising twelve populations of the Smyth line chicken. In the colony raised at the University of Florida, the amelanosis incidence has been approximately 60%. Observations compiled from four of the populations of Smyth

line chicken is summarized in Table 2-1. Female Smyth chickens (Table 2-1 and Figure 2-1) usually demonstrate a quicker onset and more severe manifestations of the phenotype of depigmentation at a higher frequency than in males, a phenomenon that has been seen in other autoimmune disease animals such as the female NOD mouse.

During the first 6-9 weeks, 42% of the females (16 of 38) became amelanotic, with 12 at the more severe levels (stages 3-5). As the birds aged, the females continued to have a higher proportion that are affected and have the more severe phenotype. By the 17th week, 66% of the females were amelanotic and of these, 20 displayed the more

Table 2-1. Amelanosis incidence in the UF Smyth line colony

Population	# number available	early onset (6-12 wks)	delayed onset (17 wks)	number amelanotic	% amelanotic	total amelanotic
SL2	6F	5/6	1/6	6/6	100%	60%
	11M	3/11	1/11	4/11	36%	10/17
SL3	14F	9/14	1/14	10/14	71%	77%
	8M	7/8	0	7/8	87.50%	17/22
SL11	9F	4/9	3/9	7/9	77%	68%
	16M	8/16	1/16	9/16	56%	17/25
SL12	17F	5/17	2/17	7/17	41%	36%
	11M	2/11	1/11	3/11	27%	10/28
				total F	72%	60%
				total M	52%	

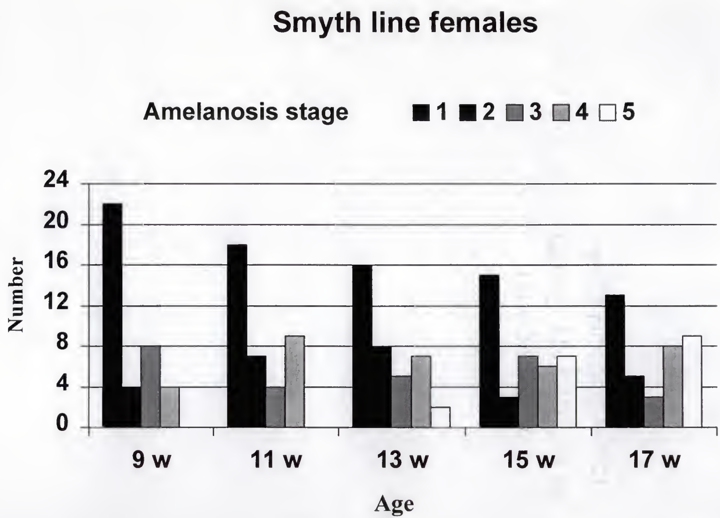

Figure 2-1. Frequencies of Smyth Line Females

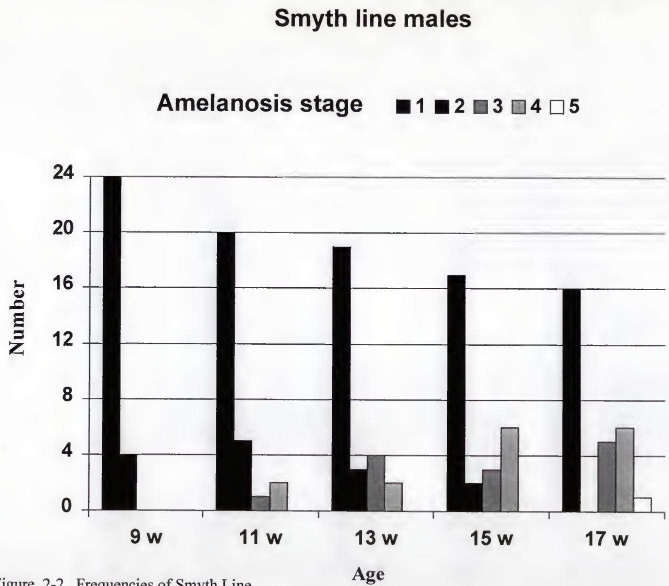

Figure 2-2. Frequencies of Smyth Line males

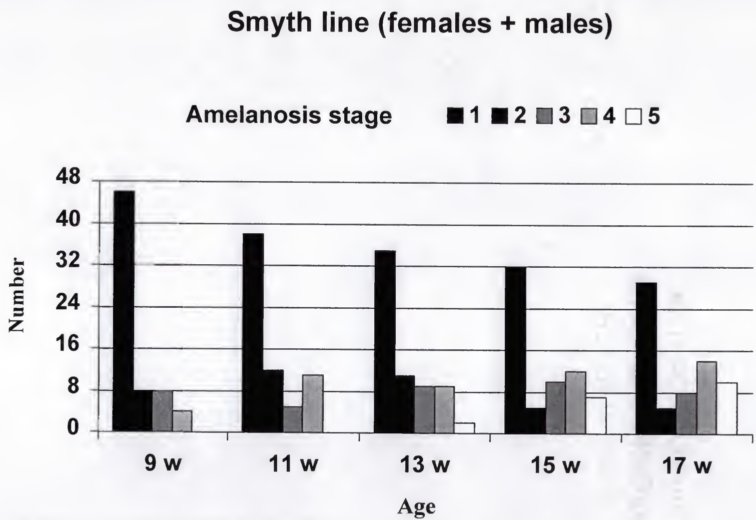

Figure 2-3. Frequencies of Smyth line females and males

severe phenotypes. Only 14% of the males (4 of 28) showed any depigmentation by the 9th week and all at the lowest level (Table 2-1 and Figure 2-2). Males can become just as severe; they usually take longer to develop. By the 17th week, 42% displayed the more severe phenotypes, but only one showed the severest at level 5, as compared to 9 females. The information combining the frequencies from both sexes is compiled in Figure 2-3. In one generation, there was actually some reversion of the phenotype in three of the females, otherwise the phenotype once the maximum was achieved remains stable. Of these three revertants two were completely amelanotic and one was 75% amelanotic. After their initial depigmentation had peaked, a period of repigmentation began, which started as patches in a different pattern than they were first depigmenting. The repigmentation was not complete.

There seems to be two waves of developing amelanosis based on observations of several populations as shown in Table 2-1 and Figure 2-3. The early onset wave (majority of the SL) begins at the 6th through the 9th week post hatch. They peaked at development of amelanosis by the 12th to 14th week, achieving the severe phenotypes, stages 4 and 5, often earlier than 12 weeks. The second wave, fully pigmented up to this point, started developing amelanosis at 17th to 20-21st week and none became totally amelanotic from the second wave. This was seen at week 17 for a male from the SL2 population and for two females from the SL12 population.

Adoptive Cell Transfer Experiments

Five cell transfer experiments were conducted. Variables considered included: (1) host age; (2) donor age; (3) stage of amelanosis exhibited by the donor (including how

rapid and severe the amelanosis developed); (4) whether hosts were irradiated; and (5) the number of injections of cells.

The first cell transfer experiment was performed on 18-19 day-old BL chicks (mean weight of 126 g). In order to allow for the possibility that changes might occur in the autoimmune T cell repertoire during the course of disease, two age groups of SL donors were used. The plan included five test groups with a one time donation of transferred cells per host, as shown in Table 2-2 (Groups 1A-1E).

Total body irradiation of 750 rads was issued per host BL bird on the day before the adoptive transfer. The splenic lymphocyte suspensions were prepared with a range of cells between $5x10^7$ cells/ml and $4x10^8$ cells/ml, which is within the range used in mouse and rat transfer experiments. Hosts and controls were kept in normal housing conditions. Of the 25 BL host chickens, 12 survived. Of these 12 BL hosts that received SL donor cells, 5 (44%) displayed a partial amelanotic phenotype. This has been summarized in Table 2-3, which shows the progression of amelanosis of these 5 hosts after receiving the transfer of SL lymphocytes.

Two females, from either groups 1A, 1D, or 1E, each developed a highly severe stage of amelanosis within 3 months of age. Unfortunately, these two died and were removed from the poultry unit by the animal caretaker before photographs and tissue samples could be taken. Unfortunate too, was the fact that the birds had outgrown their leg tags. Other BL hosts only hinted of a possible amelanotic phenotype. BL5-105, a female also from either groups 1A, 1D, or 1E, gradually developed amelanosis to stage 3 in severity. Unfortunately, this healthy bird died suddenly before photographs were considered. It too was removed from the poultry unit before samples could be taken.

Table 2-2. Adoptive transfer of amelanosis with single transfers of SL splenic lymphocytes.

Test Group	Hosts				Donors				# Amelanotic/ # Survived
	No.	Age	Sex	Irradiated (rads)	Sex	Age	Amelanosis Stage	# Cells / Host	
1A	3	18 d	F	750	F	8 w	2	5×10^7	5/12
1B	3	18 d	M	750	M	16 w	3	1×10^8	
1C	3	18 d	M	750	M	16 w	5	3×10^8	
1D	1	18 d	F	750	F	16 w	4	4×10^8	
1E	2	18 d	M,F	750	F	16 w	4	2×10^8	
2A	4	12 d	n.d.	850	M	20 w	2	7×10^8	1/4
2B	3	12 d	n.d.	850	M	32 w	(BL)	1×10^8	0/3
2C	12	12 d	n.d.	850	-	-	-	0	0/12
3A	1	6 w	F	-	M	16 w	3	1×10^9	0/1
3B	2	6 w	F	-	F	10 w	4	2×10^8	0/2
3C	2	6 w	F	-	M,F	10 w	4	2×10^8	0/2

n.d.=not determined

Table 2-3. Progression of amelanosis in 5 BL5 hosts after adoptive transfers of SL splenic lymphocytes.

		Age of host in months						
Animal	sex	3m	4m	5m	6m	7m	8m	9m
BL5	F	4 (a)						
BL5	F	4 (a)						
BL5-105	F	1	2	3 (b)				
BL5-111	M	1	2	2	3	3	3	3(c)
BL5-115	M	1	2	2	2	2	2	2(c)

(a) Died at 12 weeks old and removed from poultry unit before samples could be taken.
(b) Died within the fifth month of age and removed before photographs and samples could be taken.
(c) Died after the ninth month of age.

There are five levels to describe the degree of amelanosis as developed by Erf et al. (1995b) and adapted by this lab:

(1) normal, no apparent amelanosis; (2) mixed amelanosis, with both normal and <20% amelanotic feather tissue; (3) mixed amelanosis, with normal and 20-60% amelanotic feather tissue; (4) mixed amelanosis, with normal and >60% amelanotic feather tissue; and (5) complete amelanosis, all developing feathering tissue is amelanotic. SL chickens with stage 1 or no amelanosis are also referred to as nonprogressors, and SL chickens with any apparent amelanosis (stages 2-5) are referred to as progressors.

Two males, BL5-111 and BL5-115, also developed an amelanotic phenotype resembling that of the Smyth line and were followed more closely. Around the sixth month of BL5-111's life, regenerating feathers began to display melanocyte destruction in the pulps that had been all along reflected in the banded black and white feather vanes. Now the pulps in regenerating feathers were creamy gray instead of homogenous black. By the eighth month, the pulps in the regenerating feathers in BL5-111 were mostly gray, some were banded black and white, and only one to two percent were still completely black. Bl5-115, even in its eighth month, still displayed a low level of amelanosis with mostly black pulps in the regenerating feathers. Nevertheless, the feather vanes were banded with the oldest parts, the tips, being black and the youngest parts, closest to the pulp, being white or depigmented. The development of amelanosis was very gradual and not convincing until changes in the pulps could be witnessed. Photographs of the extent of amelanosis developed at the age of 7 months are shown in Figure 2-4 and 2-5.

In the second cell transfer experiment, three groups of 12 day-old BL7 generation hosts (sex not predetermined) were used as the recipients of a single transfer of cells comparing hosts receiving lymphocytes from a SL donor to hosts receiving lymphocytes from a BL control donor (Table 2-2).

Hosts were irradiated with 850 rad total irradiation each on the day before the adoptive transfer and given a single reconstitution of SL splenic lymphocytes or BL lymphocytes. An extra 12 BL7 which were prepared by irradiation were not used and never reconstituted with cells. These 12 survived until sacrificed after 20 plus weeks of age.

Figure 2-4. BL5-111, a Brown Line adoptive transfer host displaying stage 3 amelanosis

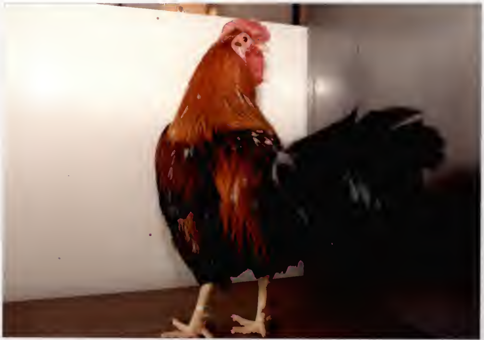

Figure 2-5. BL5-115, a Brown Line adoptive transfer host displaying stage 2 amelanosis

Only three BL7 hosts showed any manifestations of amelanosis above, BL7-222, (male), showed amelanosis of stage 2. Out of 4 host BL chickens, reconstituted with SL donor cells, only one (25%) demonstrated amelanosis. None of the control hosts reconstituted with BL donor cells developed any signs of amelanosis.

In the third adoptive cell transfer experiment, older 6 week-old non-irradiated, normal hosts were given one injection of SL donor splenocytes, as shown in Table 2-2 (Groups 3A-3C). Donor cells came from two different age groups. The change to 6 week-old chickens and no irradiation provided the opportunity of the donated SL splenic lymphocytes to be present during the time period in which amelanosis usually starts in the early onset SL chickens. None of the 10 host BL chickens developed any signs of amelanosis by the age of 20 weeks.

In the fourth cell transfer experiment, a serial adoptive cell transfer protocol was adopted. Each set of BL hosts, which were not irradiated, received several cell injections from a series of amelanotic SL donors over a course of several weeks. The transfers began at 6 weeks of age (just before the time when amelanosis begins to appear in the SL) and continued through the time period (8-16 weeks) during which amelanosis usually develops in the SL (Table 2-4, Group 4). It was hoped that weekly repeated injections of splenic cells from SL donors into the same set of BL hosts would mimic the constant presence of melanocyte-sensitized T cells in the SL chicken.

In experiment 4, 5 female BL7 hosts initially received SL donor cells representing three different experimental test groups, Groups 4A-4C. However, it was necessary to simplify the source of donor cells in the subsequent transfer of SL lymphocytes to one

Table 2-4. Adoptive transfer of amelanosis with multiple transfers of SL splenic lymphocytes.

Test Group	Hosts			Donors				#Amelantic/ #Survived
	No.	Sex	Age	Sex	Age	Amelanosis Stage	# Cells/ Host	
4A	2	F,M	6 w	M	16 w	3	$1x10^9$	
4B	1	M	6 w	F	10 w	4	$2x10^8$	
4C	2	F,M	6 w	M,F	10 w	4	$2x10^8$	
			7 w	F,F	11 w	4	$3x10^8$	0/5
			8 w	M,F	12 w	5,4	$2.7x10^8$	
			9 w	M,F,F	11 w	4,3,4	not counted	
			10 w	M,M,F	11 w	4,4,3	$1.5X10^9$	
5A	7	F	6 w	M	17 w	4	$5.6x10^8$	
			7 w	M,F	17 w	3,4	$7x10^9$	0/7
5B	3	F	6 w	M	16 w	BL	$5.5x10^9$	
			7 w	F,M,F	8 w	BL	$9x10^8$	0/3

source of donors each week. This was dictated by the availability of a large enough pool of SL donors with the desired stage of amelanosis and the work necessary to prepare the splenocytes from several SL donors. Thus if SL males provided the desired amelanotic features, their spleens were used to augment the SL donor cell source. The hosts in Group 4 were retained until 20 weeks of age; however, no amelanosis was apparent.

The fifth adoptive cell transfer experiment also utilized 6 week-old nonirradiated normal BL hosts with the same protocol as in the fourth experiment. Seven BL9 female hosts were given injections of SL donor splenic lymphocytes on week 6 and then on week 7 (Group 5A). A control group of 3 BL9 females received injections on both weeks of BL splenic lymphocytes (Table 2-4, Group 5B). These hosts were maintained for 18 weeks, and no amelanosis was observed.

Serum Transfer Experiment

We performed one experiment to determine whether the transfer of SL serum autoantibodies to melanocyte antigens could result in the transfer of the disease into BL chickens as evidenced by an amelanotic phenotype. Repetitive administrations of serum have been utilized in previous studies of passive transfer of autoimmunity. For example, in the autoimmune murine model for thyroiditis, serum transfers were performed on day 0, 2, and 4. This was sufficient to induce thyroid lesions by passive transfer of immune serum in 10-12 week old animals (Tomazic and Rose, 1975). The regiment of weekly 1-ml injections has been performed for 4 weeks by intravenous and subcutaneous means in the OS chicken model of thyroiditis (Jaroszewski et al., 1978). Serum was collected from SL chickens that expressed amelanotic phenotypes of various different stages (stages 2-5)

to account for the possible changes that occur in the antibody repertoire as amelanosis progresses. We collected and pooled serum from four different sources:

Pool 1 = 10-11 weeks old (SL11)

Pool 2 = 3-7 months old (SL6, SL7, SL10)

Pool 3 = 11 months old (SL3)

Pool 4 =17 months old (SL2)

Eight normal BL10 hosts received SL gamma globulin injections over a 4-week period. Three BL10 hosts received PBS as a control group.

A Bio-Rad protein assay was used to determined the total gamma globulin fraction administered per injection by assaying aliquots saved from each preparation used for injection. Results of this assay, (Table 2-5) indicate that the hosts received a total of 99 to 121 mg of total serum gamma globulins, which corresponds to the original serum volumes of 82 to 116 ml per host.

The experimental approach for this experiment combined elements of the aforementioned mouse (Tomazic and Rose, 1975) and OS chicken (Jaroszewski et al., 1978) studies. It included 3 administrations during the first week and then additional inoculations weekly for four additional weeks. Each autoantibody (gamma globulin) transfer consisted of the injection of a total of 5 ml per host: 2 or 3 ml into the jugular vein and 2-3 ml injected intraperitoneally (Table 2-6). Hosts and controls were kept in normal housing conditions.

These gamma globulin transfer hosts were maintained and monitored biweekly for visual changes in phenotype and regenerating feathers were collected biweekly. No

Table 2-5 BioRad Protein Assay of Gamma Globulin Pools and Selected Serum Samples

Standard (ug/ml)	OD 595	OD595	OD ave.
0	0.336	0.324	0.33
27	0.498	0.46	0.479
57	0.632	0.649	0.6405
80	0.757	0.747	0.752
107	0.825	0.864	0.8445
134	0.93	0.957	0.9435

Bovine Gamma Globulin standard
ddH_2O as reference

correlation	0.0995
slope	0.0046
y-intercept	0.3552

Sample	Dilution	OD 595	OD 595	OD ave	Dilution ug sample	Stock ug/ml	mg/5 ml	Conc. Factor	Equiv. Vol.
pool 1(sm. vial)	1/20	0.595	0.61	0.6025	53.96	1079.2	5.39	2.77	13.85
pool 1(lg. vial)	1/50	0.534	0.57	0.552	42.86	2143	10.72	2.77	13.85
SL3 pool 50ml con	1/50	0.567	0.593	0.58	48.95	2447.5	12.24	2.2	11
(SL3) pool 2 3/7/97	1/50	0.769	0.798	0.7835	93.27	4663.5	23.32	2.2	11
(SL3) pool 2 2/21/97	1/50	0.744	0.686	0.715	78.3	3915	19.58	2.2	11
(SL3) pool 2 2/26/97	1/50	0.497	0.563	0.53	38.1	1905	9.53	2.2	11
SL2 pool 3/13/97	1/50	0.822	0.899	0.8605	109.9	5495	27.48	5.61	28.05
SL2 pool 3/13/97	1/50	0.803	0.859	0.831	106.6	5330	26.65	5.61	28.05
SL2 pool 3	1/50	0.939	0.972	0.9555	130.6	6530	32.65	5.61	28.05
SL 11 pool	1/100	0.711	0.729	0.72	79.4	7940	11.91 (a)	4.3	6.45
BL5-111 4/18/97	1/50	1.035	1.043	1.039	148.9	7445	37.23		
BL5-111 5/16/97	1/50	1.073	1.167	1.12	166.5	8325	41.63		
BL5-115 4/18/97	1/50	0.963	1.026	0.9945	139.2	6960	34.8		
BL5-115 5/16/97	1/50	1.214	1.101	1.1575	174.7	8735	43.68		
SL12 1611	1/50	0.946	1.059	1.0025	140.9	7045	35.23		
SL12 1619	1/50	0.924	1.008	0.966	133	6650	33.25		
BL3 205	1/50	0.999	1.065	1.032	147.5	7375	36.88		
BL3 203	1/50	1.181	1.179	1.18	179.6	8980	44.9		

(a): mg/1.5ml

Table 2-6. Adoptive transfer of SL gamma globulin into 6 week-old BL10 hosts

Day of Injection	Age	Pool	ml	Serum Equiv.(ml)	mg IgG
1	6 w	2	5 ml	14	10.7
3	6 w	2	5 ml	14	10.7
6	6 w	2	5 ml	14	10.7
15	7 w	3	5 ml	11	18.4
23	8 w	1	1.5 ml	6	11.9
30	9 w	3	5 ml	11	18.4
		4	5 ml	28	28.9
37	10 w	3	5 ml	11	18.4
		4	5 ml	28	28.9

induced amelanosis was apparent by the age of 18 weeks at which time it was decided to terminate the experiment, 8 weeks after the last injection of gamma globulins.

Western Blot Analysis

Western blot analysis was performed to confirm the presence of antimelanocyte autoantibodies in the serum pools used for the gamma globulin adoptive transfer experiments. Serum from the two adoptive cell transfer BL hosts that became melanotic, BL5-111 and BL5-115, were also examined to learn more about the extent of their phenotype.

As shown in Figure 2-6, serum pools 1, 3, and 4 (lanes 1, 2, and 3 respectively) did contain autoantibodies specific to melanocyte proteins in the size range of 65-80kDa, reported as characteristic of the Smyth line chickens by Austin et al. (1992). Lanes 4, 5, 6, and 7 are serum samples from BL adoptive cell transfer hosts, BL5-111 and BL5-115, from two timepoints each at about a month apart. All four samples had the same autoantibody profiles specific for the same melanocyte proteins as seen typical in the other SL sera (lanes 1, 2, and 3). This suggests that, in addition to the partially amelanotic phenotype observed in these hosts, antimelanocyte autoantibody production may have been induced by the adoptive transfer of SL splenic lymphocytes. Control sera included an amelanotic SL positive control that recognized the same 65-80 kDa protein bands, and a BL serum negative control (lane 8), which did not contain autoantibodies that would recognize the same melanocyte proteins.

According to Austin and colleagues, the SL antimelanocyte antibodies recognize melanocyte proteins between 65 kDa and 80 kDa in size. Using Image Maker software,

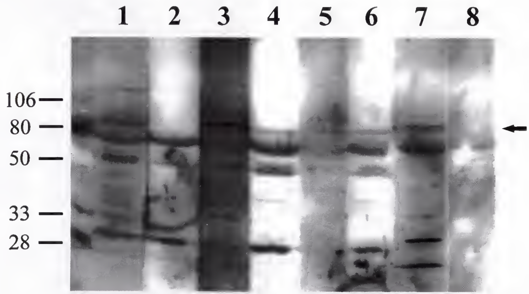

Figure 2-6. Adoptive cell transfer hosts detect the same melanocyte autoantigens between 65kDa to 80kDa as do the SL donor serum (lanes 4-7). These may be anti-Trp-1 autoantigens (Austin et al., 1992; Austin and Boissy, 1996). Gamma globulin sources (lanes 1-3) did provide serum antimelanocyte antibodies of the correct specificity and size range typical of SL chickens. Arrow refers to the detection of an approximately 79kDa melanocyte protein found only with serum antibodies from SL chickens and the adoptive SL cell transfer hosts (lanes 1-7) but not with BL chicken serum (lane 8). Bio Rad Low Range prestained markers were used.

the principal bands observed between the 49.5 and 80 kDa molecular weight markers have a size of 65 to 80 kDa proteins (Figure 2-6). The 64-65 kDa protein band is seen across all lanes (see the lower of 2 bands next to 80 kDa marker) including in the BL (lane 8).

A 79 kDa protein band was recognized by all the SL samples (lanes 1-7) but not by BL samples, which corresponds to a protein reported by Austin and colleagues as SL-specific (1992 and 1995). This figure is representative of the results of three blots using 3 different BL animals. None of the three BL control sera tested had antibodies that recognized the 79 kDa band. Austin and Boissy (1995) demonstrate that the melanocyte-specific proteins of the 65-80 kDa range are Trp-1 specific. This 79 kDa protein may therefore be Trp-1.

Regenerating feathers were plucked biweekly and collected of BL5-111 and BL5-115 starting from the fifth month. Cryosections were prepared and immunostained for CD3 expression using mouse monoclonal antibody as referenced in Chapter 1 (Erf et al. 1995b; Cooper et al., 1991). As compared to feathers from a BL control chicken, the barb ridges of feathers from BL5-111 contained a gradually decreasing amount of melanin in the barb ridges (data not shown). The last sample taken early in its last month, was completely without melanin. This suggests that in the BL5-111 host, antimelanocyte lymphocytes had penetrated beyond the confines of the pulp and invaded the barb ridges to destroy and remove the melanocytes and their products. BL5-115 samples did not show these changes and resembled the fully pigmented barb ridges of the BL control. The tissue samples were poorly preserved due to improper freezing and so can not be published. These findings, in addition to the obvious change in phenotype and the serum

antibody profile, suggest several samples of evidence of the changes induced by the adoptive transfer of SL lymphocytes in this BL5-111 host.

Discussion

Adoptive transfer experiments often can provide a means to study the autoimmune response *in vivo* in the intact organism. We used this experimental approach to determine whether melanocyte-sensitized lymphocytes and/or autoantibodies have the capacity to transfer amelanosis in a non-Smyth line chicken.

In hindsight, this portion of my project presented numerous technical challenges for a chicken animal model, including a relative lack of published protocols for adoptive transfer, radiation doses, etc. Most literature reported protocols in which mice were suppressed soon after sexual maturity (8-10 weeks). However, chickens are not sexually mature until the fifth or sixth month and that is unsuitable for the study of amelanosis in the SL. Most literature reported using approximately 5×10^7 donated cells whether the cells were bone marrow cells, spleen, or lymph node cells. Our experiments involved the transfer of up to 1×10^9 cells.

I had consulted several authorities on the chicken animal model, but never found a standard protocol for the immunosuppression of hosts of cell transfer that answered such questions as to its necessity, by which means, and how much per age or weight or other measurable parameter. Most published mouse and rat experiments did use irradiation (Wicker et al., 1986; Hanafusa et al., 1988; Panitch and McFarlin, 1977; van der Veen et al., 1989; Toivanen et al., 1975). Miller et al. (1988) subjected 4 to 8 week-old NOD mouse recipients with up to 950 rad of irradiation. However, within 2 hours the hosts

were reconstituted with splenocytes. In our experiments, the irradiation occurred the day before. Irradiation was probably necessary based on the fact that the animals in our experiments that did display induced amelanosis had been irradiated. Like et al. (1985), working on the adoptive transfer of diabetes in the biobreeding rat, concluded that an "…an intact immune system protects against adoptive transfer and diabetes ...", and so they suggested the requirement for immunosuppression. An alternative to irradiation that could have been used is cyclophosphamide (Cy) which has been used to immunosuppress the hosts in the mouse and rat (Hanafusa et al., 1988) and in the chicken (Toivanen et al., 1990; Lehtonen et al., 1975; Glick by personal communication and Glick, 1977; Olah and Glick, 1978; Glick, 1971). Transferring unfractionated splenic lymphoid cells produced higher incidence of insulitis than T cell subsets in the mouse (Hanafusa et al., 1988).

A logical next step in our experiments would be to transfer T cell subsets. As demonstrated in various studies of the subsets of T cells mediating diabetes in the NOD mouse, some researchers report the both the $CD4^+$ and $CD8^+$ T cell subsets were necessary to transfer diabetes into recipients (Miller et al., 1988; Bendelac et al., 1987). Mitsunobu et al. (1992) concluded that $CD4^+$ T cells cause the insulitis and that the $CD8^+$ T cells act as mature killer cells against the β cells with the aid of $CD4^+$ T cells. This ability to distinguish separate roles for different subsets of cells as demonstrated in the mouse should be possible in the SL chicken model for amelanosis.

Mouse monoclonal antibody reagents specific for T cells expressing $\gamma\delta$ (TCR1), $\alpha\beta V\beta1$ (TCR2) and $\alpha\beta V\beta2$ (TCR3) (Chen et al., 1988; Chen et al., 1989; Char et al., 1990) are available. The chicken homologues to CD3, CD4, CD8, CD1, CD2, CD5,

CD45, MHC class I, MHC class II, and IL-2 receptor are also recognized by mouse monoclonal antibodies (Cooper et al., 1991). Negative selection by means of complement lysis of the non-desired T cell subsets, or positive selection using mouse anti-TCR mAb and goat anti-mouse conjugated to magnetic beads are established methods. Another possibility would require the isolation of clonal populations from regenerating feathers using Con-A and IL-2 stimulated T cell subsets (Koevary et al., 1983; Panitch and McFarlin, 1977). This would most closely resemble the T cells at the site of melanocyte destruction (Erf et al., 1995b). There is the issue of the unknown feasibility of adopting this technique to the chicken *in vivo*.

Nevertheless, the fact that 5 out of 12 BL5 hosts (44%) and 1 out of 4 BL7 hosts (25%) survived long enough to manifest amelanosis by adoptive cell transfer indicates that the amelanosis found in the SL chickens may be cell-mediated. A total of 40 BL hosts of SL splenic lymphocytes were followed from all five experiments and of these 6 hosts (15%) became amelanotic. This is not by chance considering BL chickens normally demonstrate only 1-2% comparatively. These results suggest that the autoreactive lymphocytes are capable of recognizing BL, as well as SL, melanocytes, and that the SL melanocyte defect may not be required for pathogenesis.

The T cell-mediated melanocyte destruction in vitiligo would resemble the T cell-mediated destruction of pancreatic β cells in diabetes, demyelinization in myelin basic protein-induced EAE, and destruction of follicular epithelial cells in autoimmune thyroiditis. T cell involvement has also been suggested in two other autoimmune chicken conditions, hereditary scleroderma in the UCD-200 and UCD-206 lines (Haynes and

Gershwin, 1983; van der Water et al., 1989) and thyroiditis found in the Obese strain (OS) chicken (Brown et al., 1991; Wick et al., 1970). The observation that adoptive transfer of SL lymphocytes may cause amelanosis in BL hosts is significant, in part because previous authors on the amelanosis of the SL chicken have over-emphasized the role of autoantibody, based on studies of bursectomy and corticosteroid inhibition of amelanosis (Lamont and Smyth, 1981; Boyle et al., 1987).

It is also interesting to note that the western blot analysis demonstrated that the BL adoptive cell hosts had the same antimelanocyte serum antibody profile as the typical SL serum. This result suggests that either: (1) melanocyte-reactive SL B cells were transferred; or (2) melanocyte-reactive SL T helper cells induced BL B cells to produce anti-melanocyte autoantibodies.

One must consider the possibility that the BL hosts that became amelanotic as a result of the cell transfers may actually have been susceptible to amelanosis given the 1 to 2% incidence of amelanosis in the BL. The introduction of autoreactive SL lymphocytes may have "tipped the scale" in favor of disease in these individuals. This is the conclusion that adoptive transfer of splenocytes in the NOD mice accomplished in the work by Wicker et al. (1986).

The repetitive administrations to the BL hosts with the transfer of SL-sensitized T cells or autoantibodies during the time period during which amelanosis has normally occurred in the SL donors appeared to be a logical approach to inducing amelanosis and should be done in combination with immunosuppression. Future studies should include these two conditions. Recall that in the first experiment the BL5 hosts were immune suppressed and were given one transfer of cells, resulting in five BL5 recipients that

displayed the amelanotic phenotype to some degree. The serial transfer experiments (3 and 4) with older (6-week-old) not-immune-compromised hosts were given up to 5 repeated transfers of donated SL cells. Still they had not displayed a hint of amelanosis in a situation we anticipated would have an earlier onset. Future experiments should involve both immunosuppression by irradiation and serial transfers.

Previous attempts to induce an autoimmune disease by injection of antisera in normal chickens have been unsuccessful (Jaroszewski et al., 1978). Anti-thyroglobulin antisera from OS chicken suffering from thyroiditis were transferred to the normal Cornell strain but failed to induce thyroiditis, despite the fact that they share the same B alleles at the MHC locus. Immunocompromising these Cornell recipients did not help, even though neonatal thymectomy did potentiate the severity of thyroiditis in OS chickens (Wick et al., 1970), as immunosuppression seemed to have done for our cell transfer experiments.

Timing of the transfers may have an influence on the success. Wicker et al. (1986) noticed that they were unable to transfer diabetes using splenocytes if the NOD irradiated mice were less than or equal to 6 weeks old, but were much more effective with transfers to hosts that were slightly older than 6 weeks of age. For the Smyth line chicken, perhaps the transfers needed to be initiated earlier for both our cell and humoral administrations. Perhaps *in vitro* stimulation of the splenocytes with lectins or melanocyte fragments in the donor may help (Takenaka et al., 1986; McCarron and McFarlin, 1988). Cell transfers have been successful from bone marrow and mature splenocytes or lymph node cells. Our utilization of repeated transfers of cells or gamma globulin, and the concentration of the immunoglobulins used should have addressed the

problem of maintaining high enough concentrations of effector cells or antibodies over long enough periods of time, a problem encountered by others in serum transfer experiments (Inoue et al., 1993).

In summary, the experiments involving the adoptive transfer of SL splenocytes into the BL hosts suggests that amelanosis can be transferred and may be a cell-mediated autoimmune process. The role of the associated autoimmune antibodies may not be as clear. Whether autoantibodies can cause or trigger the destruction of the melanocytes in BL hosts was not apparent. In diabetes, autoimmune antibodies against islet cells is a distinguishing feature of the disease, precedes the onset of the disease, are specific to the 65kDa autoantigen GAD, and can be used as predictive markers of patients susceptible to the disease. This likewise may be a similar situation with the autoantibodies in the SL chicken. The role of the autoantibodies in diabetes as a primary inducer of diabetes has not yet been proven.

CHAPTER 3

T CELL RECEPTOR γ REPERTOIRE ANALYSIS OF THE EXPANDED PERIPHERAL BLOOD $\gamma\delta$ T CELL POPULATION DURING AVIAN VITILIGO

Introduction

Many autoimmune diseases are T cell-dependent, which, in part, can be determined by the study of TCR genes. T cell receptors can generate up to 10^{16} total receptor specificities by combinatorial and junctional diversity (Janeway and Travers, 1997), although much of this diversity is lost during thymic education during the induction of central tolerance. During an immune response, T cells respond in a clonal fashion due to TCR recognition of antigenic peptides. Similarly, during an autoimmune response, a fraction of T cells may be clonally expanded in response to self-antigen. The T cell response may be described as (1) polyclonal, in which many T cell clones are recruited in the response, (2) oligoclonal, in which a small number of T cell clones expand, or (3) monoclonal, in which one specific clone responds. Experimentally, this can be studied by TCR repertoire analysis of a T cell population. The identification of recurrent TCR sequences (with the same or similar CDR3) in large T cell populations provides evidence for antigen-driven expansion.

T cells present in acute graft-versus-host-disease of target organs (skin, liver, and intestine) in HLA-matched allogeneic bone marrow transplants express predominantly

the $\alpha\beta$ TCR (Dietrich et al., 1994). The TCR Vα and Vβ usage in both skin and blood appeared unrestricted, but with overexpression, unique to each patient, of a few Vα and Vβ gene segments in the skin as compared to blood. To avoid the possibility that a nonspecific inflammatory response would mask detection of a clonal T cell expansion, Dietrich and colleagues concentrated on one patient. Evidence for the repeated usage of at least five specific TCR Vα11 and Vβ16 transcripts, with CDR3 of unique length and sequence, indicated an oligoclonal expansion in the skin, with specific V genes overexpressed as compared to the blood, which also showed some of the same recurrent TCR transcripts but to a lesser degree.

Even when genetically identical mice are raised in the same environment, murine intestinal intraepithelial lymphocytes (IEL) display unique oligoclonal repertoires. As compared to lymph node, Vβ expression of $\alpha\beta$ TCR$^+$ IELs is oligoclonal, as indicated by prominent and distinct subsets of clonal populations of IELs detected as peaks in their CDR3 length analysis (Regnault et al., 1994). This contrasted with the polyclonal repertoire of respective T cells in the lymph nodes.

Likewise, human IELs of five patients were compared (Blumberg et al., 1993). The dominant Vβ of the IELs in patient 1 was Vβ3 and every Vβ3 isolated was identical. The dominant Vβ in patient 2 showed one Vβ13, two Vβ5 clones, and an oligoclonal expansion of Vβ4 and Vβ6. Patient 3 displayed either monoclonal or oligoclonal dominant Vβ gene expression. This was in contrast to a totally polyclonal TCR repertoire in the PBL sampling, where no sequence was repeated. Therefore, one or a small number of dominant clones appear to comprise the majority of IEL. The conclusions are that

most IELs are clonally expanded, express a small number of different Vβ genes, and may recognize a limited number of antigens. Blumberg and colleagues suggest that this small T cell repertoire would suffice if the target antigens were conserved, such as bacterial heat shock proteins, or a small number of endogenous antigens expressed by IELs in response to injury. Similar to the report of Dietrich et al. (1994) on human T cell repertoires, the IEL clonal expansion is unique to each individual; there were no shared clonal sequences.

Kourilsky and co-workers applied their high resolution PCR-based method of determining and following the TCR repertoire in heterogeneous cell populations of tumor-infiltrating lymphocytes (TILs) in human melanomas. Results confirmed clonal expansions in a rather complex polyclonal background. Detection of clonal T cell expansions before, during, and after treatment is facilitated using this method of PCR-detection of junctional diversity in terms of CDR3 length and sequence clonality (Puisieux et al., 1994).

In the Smyth line chicken, the melanocytes, which are located in developing feathers and the choroid layer of the eye, appear to be the target of both T and B cell-mediated autoimmune responses (Smyth, 1989). Cryosections of the regenerating feathers display an intense T cell infiltration including both αβ and γδ T cell lineages (Erf et al., 1995b). In addition, γδ T cells are found in increased proportions in the peripheral blood of 40 week-old SL chickens (44.1%) as compared to MHC-matched parental BL chickens (33.2%) (Erf et al., 1996) This observation is especially intriguing given the fact the chickens have a significantly greater proportion of γδ T cells within the PBL

compartment than humans and mice, e.g., 20-50% in chicken and 3-5% in human and mouse (Bucy et al., 1988). One possible explanation for the expansion of PBL γδ T cells is that melanocyte autoantigens caused an antigen-driven clonal expansion in the target tissues. The peripheral blood with its higher proportion of γδ T cells, may reflect this clonal expansion of γδ T cells infiltrating the feathers in the SL chicken, either as a "spill-over" effect from the target tissues. This was observed in the representation of certain Vα11 and Vβ16 TCR transcripts in the blood reflecting the "spillover" of T cells infiltrating the skin during human GVHD (Dietrich et al., 1994).

γδ T cells represent only 0.5-10% of the normal mammalian peripheral blood T cell population, and they are believed to be involved in immunity to infectious disease (Haas et al., 1993; Modlin et al., 1993; Mombaerts et al., 1993). A role for γδ T cells in the pathogenesis of autoimmune disease has been suggested due to their reactivity to stress proteins (Kaufman, 1990) and by the accumulation of γδ T cells in affected organs and peripheral blood. For example, in systemic sclerosis, γδ T cells expressing the TCR $V\delta1^+$gene segment are expanded in both PBL and the lungs, and have restricted junctional diversity in terms of CDR3 length and sequence (Yurovsky et al., 1994 and 1995). This was indicated by a significantly higher proportion of repeated sequences in the patients than in controls suggesting that $V\delta1^+\gamma\delta$ T cells may be Ag-driven in systemic sclerosis patients. The percentage of γδ T cells is expanded in PBL of patients with inflammatory bowel disease, showing some skewing of Vδ gene expression (Bucht et al., 1995). Similarly, the frequency of γδ T cells is higher in PBL and cerebrospinal fluid of multiple sclerosis patients, as compared to other neurologic disease patients and normal

individuals, with preferential Vγ and Vδ gene expression, but heterogeneous clonal origins (Stinissen et al., 1995). Systemic lupus erythematosus patients are reported to express a diverse PBL Vγ repertoire, but an oligoclonal Vγ repertoire restricted in terms of Vγ gene usage and junctional diversity (Olive et al., 1994). Finally, increased levels of peripheral blood γδ T cells have been correlated with increased risk for insulin-dependent diabetes (Lang et al., 1991). Taken together, these reports suggest a possible role for γδ T cells in the development of autoimmune diseases or associated inflammatory processes.

We tested the hypothesis that the expanded PBL γδ T cell repertoire is involved in the pathogenesis of vitiligo in the chicken animal model by characterizing the expressed TCR-γ repertoire by nucleotide sequence analysis of Vγ genes expressed in PBL of MHC-matched SL chickens with active vitiligo and control BL chickens. The chicken TCR-γ locus consists of three families of 8-10 Vγ genes, 3 Jγ genes, and a single Cγ gene segment (Six et al., 1997), thus allowing TCR Vγ repertoire analysis in the chicken animal model.

Materials and Methods

Animals

Breeding colonies were established for the B^{101} subline of the SL and BL from fertile eggs generously provided by J. Robert Smyth, Jr. (University of Massachusetts at Amherst). Chickens were raised at the University of Florida Poultry Science Unit. The SL and BL chickens used in this study were MHC-matched and all of the B^{101} subline, which is characterized by the more severe form of vitiligo in this animal model. The degree of pigment loss (amelanosis) was classified according to the following scale after

Erf et al. (1995b): (1) normal, no apparent amelanosis; (2) mixed amelanosis, with both normal and <20% amelanotic feather tissue; (3) mixed amelanosis, with normal and 20-60% amelanotic feather tissue; (4) mixed amelanosis, with normal and >60% amelanotic feather tissue; and (5) complete amelanosis, all developing feathering tissue is amelanotic.

RT-PCR and cloning

Peripheral blood lymphocytes were isolated from heparinized blood samples collected from the brachial vein by Ficoll-Hypaque density centrifugation. Total RNA was prepared using RNeasy columns (Qiagen Corp.), and eluted in RNase-free distilled water. Total cDNA was synthesized using random primers and Superscript-II reverse transcriptase according to the supplier (Gibco-BRL).

Rearranged chicken Vγ genes were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (Six et al., 1996) using the following Vγ family-specific forward primers and a Cγ reverse primer:

Vγ1 5'-GCTCTAGACTGAAGCCTGGTTGCATCC-3'

Vγ2 5'-GCTCTAGACCCATACAGAGCCCTGTATCC-3'

Vγ3 5'-GCTCTAGAGCAGACAACATGCTGCTG-3'

Cγ 5'-CCTGGATCCTTTCATAATTCTCTGGTGCTG-3'.

PCR reactions were performed in 50 μl with 2.5 units Taq polymerase and buffer provided with the enzyme (Boehringer Mannheim Biochemicals), cDNA template, 0.2 mM dNTPs, and 20 pmoles of each primer, for 28 cycles of 60 sec at 94°C, 75 sec at 55°C, and 90 sec at 72°C. RT-PCR products were cloned into the vector pBluescript-II

(Stratagene) using restriction sites built into the PCR primers, or cloned into pNoTAT7 using a Prime PCR Cloner cloning kit (5 Prime 3 Prime, Inc.) by blunt-end ligation. Clones were selected by blue/white selection and by size analysis of amplified inserts by agarose gel electrophoresis. Individual clones were sequenced using dideoxynucleotide cycle-sequencing kits (Applied Biosystems, Incorp.) and T7 and T3 primers.

Nonincorporated dye terminators were removed on Centri-Sep spin columns (Princeton Separations, Adelphia, NJ), and reactions were analyzed on an Applied Biosystems Model 373A DNA sequencer. Chromatograms were analyzed using Applied Biosystems software.

DNA sequence comparisons

For sequence comparisons, only sequences with open reading frames were included in the alignments. We have recently described reference sequences for the Vγ1, Vγ2 and Vγ3 families, as well as individual Vγ family members (Six et al., 1996). The ALIGN Plus program (version 2.0, Scientific & Educational Software) was used for initial sequence alignments.

Results

Phenotype of birds used for repertoire analysis

The phenotypes of the SL chickens selected for this study are shown in Table 3-1.

Table 3-1. Amelanosis Stage of Smyth Line Chickens at Ages 2-25 Weeks

Animal #	2w	5w	10w	15w	20w	25w
S1	1	3	4	5	5	5
S2	1	1	3	4	4	4
S3	1	3	4	5	5	5
S4	1	1	4	4	5	5

Two birds (S1 and S3) were characterized by early onset of amelanosis, prior to 5 weeks of age, and three of four SL birds were completely amelanotic at the time of PBL collection at 25 weeks of age. As controls, PBL were used from two age-matched BL chickens, which had normal plumage.

TCR Vγδ repertoire analysis

Rearranged Vγ1, Vγ2 and Vγ3 genes were amplified by RT-PCR from PBL mRNA, cloned into a plasmid vector, and individual clones were sequenced. A total of 86 rearranged Vγ genes were sequenced from the PBL of BL and SL chickens. Thirteen sequences were eliminated due to the presence of stop codons or frame-shifts in CDR3. The presence of these clones was not unexpected, because the PBL were not pre-enriched for γδ T cells, and nonproductive TCR-γ gene rearrangements would be expected in the total PBL population from αβ as well as γδ T cells. The first indication of the heterogeneous clonality of the cloned Vγ genes from both BL and SL was indicated by the fact that only 5 sequences were repeat sequences, i.e. identical to other clones derived from the same animal.

Partial nucleotide sequences for the Vγ framework region 3 (FR3), complementarity determining region 3 (CDR3) (*i.e.* the Vγ-Jγ junction), and FR4 (encoded by the Jγ gene segment), are shown in Figures 3-1, 3-2, and 3-3 for Vγ1, Vγ2, and Vγ3 families, respectively. BL and SL Vγ sequences are aligned with reference sequences we have recently described for Vγ gene families in White Leghorn (WL) chickens (Six et al., 1996). Identical sequences were obtained from different animals in only two cases. Two identical Vγ2 sequences were obtained from two different BL

```
                                                                             Asp         TyrTyrCys
Vγ1-186       TATACATCTTCCAAAATGGGGAATAAAATCTGCACTCTCTCAGTTCAAGACATAGGTGATGATGATAAAGGTACCTACTACTGT
Vγ1-B2.17     ....................................................................................
Vγ1-B2.24     ....................................................................................
Vγ1-B2.20     ....................................................................................
Vγ1-B2.15     ...TTG....AT....GT.A.....TG.........................................................
Vγ1-B2.21     ...TTG....AT....GT.A.....TG.........................................................
Vγ1-B2.18     ...TTG....AT....GT.A.....TG.........................................................
Vγ1-B2.2      ...TTG....AT....GT.A.....TG.........................................................
Vγ1-B2.22     ...TTG....AT....GT.A.....TG.........................................................
Vγ1-S2.1      ....................................................................................
Vγ1-S2.3      ......................................................C...........G.................
Vγ1-S2.5      ......................................................C...........G.................
Vγ1-S2.2      ......................................................C...........G.................
Vγ1-S1.1      ......................................................C...........G.................
Vγ1-S1.4      ......................................................C...........G.................
Vγ1-S1.6      ......................................................C...........G.................
Vγ1-S6.3      ......................................................C...........G............G....
Vγ1-S1.7      ..........................C...........................C...........G.................
Vγ1-S1.2      ...TTG....AT....GT.A.....TG.........................................................
Vγ1-S2.11     ...TTG....AT....GT.A.....TG.........................................................
```

```
                                   N region                                 PheGly   Gly                         Jγ
Vγ1-186      GCCTACTGGGAGTC     ACGAT       CCGGATATTACTACAAAGTTTTCGGCTCTGGTACAAAGCTCATTGTATCAGAC  1
Vγ1-B2.17    ......             GCTTCTA           CA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ1-B2.24    ......             GTCGTA     TATA.TGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ1-B2.20    ......             CCGG           ATGG.G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ1-B2.15    .......                           ATGG.G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ1-B2.21    ..............T    ATATCGGTTG  GAT.......................................C.............  1
Vγ1-B2.18    .......                        .....................................................  1
Vγ1-B2.2     .............      TAC            ..................................................  1
Vγ1-B2.22    .............      TAAGACCTG   GAT.....................................................  1
Vγ1-S2.1     ......A.......     CGTATACGG                TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ1-S2.3     ....             GGTGGAGAGGCAT          A.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ1-S2.5     .........          ATCA                  ...............................................  1
Vγ1-S2.2     ...........A..     TTGG                    .............................................  1
Vγ1-S1.1     ..........         GAGGTA      .....................................................  1
Vγ1-S1.4     ...........        TC          GAT.....................................................  1
Vγ1-S1.6     ............       CGGGCCC     GAT.....................................................  1
Vγ1-S6.3     ...........        TCGC                    .............................................  1
Vγ1-S1.7     ......             GTCAAC           GG.G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ1-S1.2     ..........         TT            ...................................................  1
Vγ1-S2.11    .......            CTTTGGGG         ................................................  1
```

Figure 3-1. Partial nucleotide sequences of rearranged TCR-Vγ1 genes from Brown line and Smyth line chickens, showing the FR3, N region and Jγ sequences. Clone designations include an animal number (shown in Table 3-1 for SL) and an individual clone number. Sequences recovered more than once are indicated by the number of repeats in parentheses after the clone number. Sequences are aligned with reference Vγ genes (Six et al. 1996). Evolutionarily conserved amino acids in Vγ and Jγ are indicated above the nucleotide sequence. Dots symbolize identity to the reference sequence. Nongermline encoded nucleotides are labeled as the N region. Possible P nucleotides are underlined.

```
                                                                            Asp         TyrTyrCys
Vγ2-5029     AGGATTCAAACTATTTCTACACAGAGACTTTGCACTCTTACAATAAGAAATGTAATTCCGGATGATGATGCTACTTACTACTGT
Vγ2-B2.8     ....................................................................................
Vγ2-B2.12    ....................................................................................
Vγ2-B2.6     ...................................................................C................
Vγ2-B1.2 (3) ...................................................................C................
Vγ2-B1.7     ...............C...................................................C................
Vγ2-B1.6     .........................AG........................................C................
Vγ2-B1.5     .........................AG........................................C................
Vγ2-B2.13    .........................AG........................................C................
Vγ2-B1.4     ...................G.....AG........................................C................
Vγ2-B1.21    .C.................G.....AG........................................C................
Vγ2-B1.9     ...GC..TGTA.GGG....G.....AG............T...........................C................
Vγ2-B1.18    ...GC..TGTA.GGG....G.....AG............T...........................C................
Vγ2-S1.1 (3) ...................................................................C................
Vγ2-S6.1     ...............C...................................................C................
Vγ2-S2.13    ...............C...................................................C................
Vγ2-S1.3     ...............C...................................................C................
Vγ2-S1.4     ...................G...............................................C................
Vγ2-S2.1     .........................AG........................................C................
Vγ2-S2.9     .........................AG........................................C................
Vγ2-S1.8     .........................AG........................................C................
Vγ2-S2.10    .........................AG........................................C...........T....
Vγ2-S6.4     .........................AG........................................C...........T....
Vγ2-S1.9     .........................AG........................................C...........T....
Vγ2-S1.2     .........................AG........................................C...........T....
Vγ2-S1.6     ...................G.....AG............T...........................C................
Vγ2-S1.14    ...................G.....AG............T...........................C................
Vγ2-S6.7     .C.................G..G..AG............T...........................C................
```

```
                                   N region                          PheGly   GlyThr                     Jγ
Vγ2-5029      GCCTACTGGGACCCT     AGTCCGAGG          TATTACTACAAAGTTTTCGGCTCTGGTACAAAGCTCATTGTATCAGAC  1
Vγ2-B2.8      .....                CTCGAGA        GGA...............................................  1
Vγ2-B2.12     ............... AGTTCCTTCCCTAAGGGTACT           ....................................  1
Vγ2-B2.6      ...............      AGTGCG            ...............................................  1
Vγ2-B1.2 (3)...............      AGTGCG            ...............................................  1
Vγ2-B1.7      ...............      TCGTCC              G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ2-B1.6      ...............     GTCCTGTTCG          .............................................  1
Vγ2-B1.5      ...                 GCTCAAAA          A..............................................  1
Vγ2-B2.13     ...............       AGG                ...........................................  1
Vγ2-B1.4      ..............       CAAG         AGTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-B1.21     ...............     ATCTCTATC        GCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-B1.9      ..............       GTAC            GCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-B1.18     ...............                   AGTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S1.1 (3)...............      AGTGCG            ...............................................  1
Vγ2-S6.1      ...............      ACG                      ....................................  1
Vγ2-S2.13     ............         TCGGC            A..............................................  1
Vγ2-S1.3      ...                  CATG      GATCCGGA..............................................  1
Vγ2-S1.4      ...............       A              GA.......................................T......  1
Vγ2-S2.1      ...............     ATTAGGA            ........................................C......  1
Vγ2-S2.9      ............        TCGTGGGTTG  GATCCGGA..............................................  1
Vγ2-S1.8      ..............       GTA            TGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S2.10     ...............      ATGTCG          GCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S6.4      ...............    ATAACTCCACGGG            T....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S1.9      .......           CTCGCGTCGTCTTA  TATAGTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S1.2      .............         TA            GGA.............................................  1
Vγ2-S1.6      ...............       TCG                 .GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S1.14     ...............       CGA         TATAGTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ2-S6.7      ............... ATCGGCGGTGGGTCG          G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
```

Figure 3-2. Partial nucleotide sequences of rearranged TCR-Vγ2 genes from Brown line and Smyth line chickens, showing the FR3, N region and Jγ sequences. Clone designations include an animal number (shown in Table 3-1 for SL) and an individual clone number. Sequences recovered more than once are indicated by the number of repeats in parentheses after the clone number. Sequences are aligned with reference Vγ genes Six et al 1996). Evolutionarily conserved amino acids in Vγ and Jγ are indicated above the nucleotide sequence. Dots symbolize identity to the reference sequence. Nongermline encoded nucleotides are labeled as the N region. Possible P nucleotides are underlined.

```
                                                                                   Asp          TyrTyrCys
Vγ3-6439    AAGTTTCAAGTGCAGAGAAATCCTTCCAACTCTGTTCTGACAATAAAGAAATCAACTCGGAGGGATACCGGTACTTACTATTGT
Vγ3-B2.6(2) ....................................................................................
Vγ3-B2.1    ....................................................................................
Vγ3-B2.5    ....................................................................................
Vγ3-B2.8    ....................................................................................
Vγ3-B2.3    .....................................................T...A..........................
Vγ3-B2.2    .....................................................T...A..........................
Vγ3-B2.7    .....................................................T...A..........................
Vγ3-B1.14   .....................................................T...A..........................
Vγ3-B1.16   .....................................................T...A..........................
Vγ3-B1.13   ........................................T.....C......T...A..........T...........C...
Vγ3-B2.4    ...A....................................T.C...C.........C..A........T...........C...
Vγ3-S6.2    ....................................................................................
Vγ3-S1.13   ....................................................................................
Vγ3-S6.3    ...........................................................................C........
Vγ3-S2.8    .....................................................T...A..........................
Vγ3-S2.10   .....................................................T...A......................C...
Vγ3-S6.1    ........................................T.....C......T...A..........T...........C...
Vγ3-S3.3    ........................................T.....C.........C..A....................C...
Vγ3-S3.5    .....................G..................T..........A....C..A........T...............
Vγ3-S3.6    .....................G..................T..........A....C..A........T...............
Vγ3-S6.4    .....................G..................T..........A....C..A........T...............
Vγ3-S2.12   .....................G.....G..A.........T..........A....C..A........T...............
```

```
                                    N region                              PheGly   Gly                     Jγ
Vγ3-6439     GCATACTGGTATAAGC     CACGACGGTT     ATATTACTACAAAGTTTTCGGCTCTGGTACAAAGCTCATTGTATCAGAC  1
Vγ3-B2.6(2)  ..............          ACA    ATCCGG..............................................  1
Vγ3-B2.1     ................     GGAGTGCATGGATC                TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-B2.5     ..............          TTACC       GTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-B2.8     .........               CGA        GG..............................................  1
Vγ3-B2.3     ................AA      CA               .G.TG.G..GA.A..T..AAG...A..T..A..T.....T......  2
Vγ3-B2.2     ............T.T.AAGGC       TGGTCCGA...............................................  1
Vγ3-B2.7     ............T.T.AAGGC              CG..............................................  1
Vγ3-B1.14    ............T.T.AA      CTCA            CA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-B1.16    ............T.T.AAGGCAT ATACGGGG        ..............................................  1
Vγ3-B1.13    ............           GAAGTG              ............................................  1
Vγ3-B2.4     ............     GAACAAGGCTTCCCGA CCGG..............................................  1
Vγ3-S6.2     ................AAG       A      CTATAGTGCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-S1.13    ................AAGG    CTTAA          G..............................................  1
Vγ3-S6.3     ................AAGGC TCCCCCTCTT      ATGG.G.TG.G...A.A..T..AAG...A..T..A..T.....T......  2
Vγ3-S2.8     ................AA      CA               .G.TG.G..GA.A..T..AAG...A..T..A..T.....T......  2
Vγ3-S2.10    ............            GCCC          G..............................................  1
Vγ3-S6.1     ..............        TCCCTTGGAA        GCA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-S3.3     ............            GAAC            CA.GGAT....TAC......A.A..A..T.....G.....C..CA..  3
Vγ3-S3.5     .............G        GCAAGCCTATATC                    ......A.A..A..T.....G.....C..CA..  3
Vγ3-S3.6     .............G        GCAAGCCCGA                       .................................  1
Vγ3-S6.4     .............G          GCAA                    GAG...A.A..T..AAG...A..T..A..T.....T......  2
Vγ3-S2.12    .............G..AAG   CCTTAAGCGA          .G.TG.G..GA.A..T..AAG...A..T..A..T.....T......  2
```

Figure 3-3. Partial nucleotide sequences of rearranged TCR-Vγ3 genes from Brown line and Smyth line chickens, showing the FR3, N region and Jγ sequences. Clone designations include an animal number (shown in Table 3-1 for SL) and an individual clone number. Sequences recovered more than once are indicated by the number of repeats in parentheses after the clone number. Sequences are aligned with reference Vγ genes (Six et al. 1996). Evolutionarily conserved amino acids in Vγ and Jγ are indicated above the nucleotide sequence. Dots symbolize identity to the reference sequence. Nongermline encoded nucleotides are labeled as the N region. Possible P nucleotides are underlined.

animals (clones Vγ2-B2.6 and Vγ2-B1.2), and identical Vγ3 sequences were obtained from a BL and SL animal (clones Vγ3-B2.3 and Vγ3-S2.8).

Vγ1. All eight of the rearranged Vγ1 genes from BL and three of eleven V1 genes from SL were identified as known V1 family members based on their predicted FR3 amino acid sequence. The remaining eight of eleven Vγ1 genes from SL may represent a novel Vγ1 family member, which shares two amino acid substitutions (Gly85Arg and Lys89Glu) with three other Vγ1 genes. Considering the relatively low number of amino acid substitutions compared to WL Vγ1 genes and the absence of further genomic mapping and sequence data for the chicken TCR-γ locus, these novel SL Vγ1 genes may also represent allelic differences between known Vγ1 family members segregating in the WL and SL strains.

Vγ2. Ten of twelve BL Vγ2 genes and eight of fifteen SL Vγ2 genes could be identified as known Vγ2a subfamily members. The remaining two of twelve BL Vγ2 sequences represent a novel Vγ2 subfamily, which we designate Vγ2d, and the remaining seven of fifteen SL sequences represent three different novel Vγ2a genes, which may represent new Vγ2a subfamily members or allelic polymorphisms between WL and SL.

Vγ3. Ten of eleven BL and eight of eleven SL Vγ3 genes could be identified as known Vγ3 family members. The remaining one of eleven BL and three of eleven SL sequences represent two different novel Vγ3 genes, which may represent new Vγ3 family members or allelic polymorphisms between WL and SL. There are four SL Vγ3 sequences representing a Vγ3 member not seen in BL sequences, suggesting a possible shift in Vγ3 usage in SL as compared to BL.

CDR3 length and amino acid composition

The amino acid sequences encoded by the BL and SL Vγ genes are shown in Figure 3-4 for the region between the conserved Cys94 and Phe108 residues in Vγ and Jγ, respectively. Sequences were compared for overall CDR3 length, measured as the number of amino acids between these two anchor positions, the number of nongermline-encoded amino acids in the N region, and for the amino acid content of CDR3 and N regions. No differences were found between BL and SL for the CDR3 or N region length of all Vγ sequences combined or for individual Vγ families. When the SL Vγ amino acid sequences in the CDR3 were compared to BL, there were fewer nonpolar and more charged amino acids in Vγ1 sequences, and more nonpolar and fewer polar amino acids in Vγ3 sequences. However, none of the differences in amino acid content (frequency of nonpolar, polar, and charged amino acids between BL and SL Vγ sequences were statistically significant as determined by χ^2 analysis (data not shown).

Jγ usage

Overall the usage of the three Jγ gene segments in the BL and SL sequences was 58% vs. 51% usage of Jγ1, 13% vs. 19% of Jγ2, and 30% vs. 29% of Jγ3. Although there appeared to be more Vγ1-Jγ1 and Vγ3-Jγ2 and fewer Vγ1-Jγ3 and Vγ3-Jγ1 combinations in SL chickens as compared to BL chickens, the differences in Jγ usage overall and within each Vγ family are not statistically significant by χ^2 analysis (data not shown).

Discussion

The function of γδ T cells in general is less well understood than that of αβ T cells, and they are characterized by functional differences, such as antigen recognition

Vγ1

	Vγ	N region	Jγ
Vγ1-186	CAYWES	R	SGYYYKVF
Vγ1-B2.17	...	AST	WI.Y.
Vγ1-B2.24	...	VV	YSAWI.Y.
Vγ1-B2.20	...	PD	GDE.I.
Vγ1-B2.15	...		YGDE.I.
Vγ1-B2.21	......	ISVG	....
Vγ1-B2.18	..	S	....
Vγ1-B2.2	.....	LR	......
Vγ1-B2.22	.....	LRPGS	....
Vγ1-S2.1	...R..	VYG	E.I.
Vγ1-S2.3	..	WWRGI	WI.Y.
Vγ1-S2.5	....	IN	.....
Vγ1-S2.2	......	W	.....
Vγ1-S1.1	....	GGT	....
Vγ1-S1.4	....	DR	....
Vγ1-S1.6	.....	RAR	....
Vγ1-S6.3	....	DR	.....
Vγ1-S1.7	...	VN	GDE.I.
Vγ1-S1.2	....	V	....
Vγ1-S2.11	...	SLG	......

Vγ2

	Vγ	N region	Jγ
Vγ2-5029	CAYWDP	SPR	YYYKVF
Vγ2-B2.8	...	SR	G......
Vγ2-B2.12	......	SSFPKGT	...
Vγ2-B2.6	......	SA	......
Vγ2-B1.2	......	SA	......
Vγ2-B1.7	......	SS	DE.I.
Vγ2-B1.6	......	VLFD	.....
Vγ2-B1.5	..	AQK	......
Vγ2-B2.13	......	R	......
Vγ2-B1.4	......	R	SAWI.Y.
Vγ2-B1.21	......	ISI	AWI.Y.
Vγ2-B1.9	......	Y	AWI.Y.
Vγ2-B1.18	......		SAWI.Y.
Vγ2-S1.1	......	SA	......
Vγ2-S6.1	......	T	...
Vγ2-S2.13	.....	SA	......
Vγ2-S1.3	..	HG	SG......
Vγ2-S1.4	......	R	......
Vγ2-S2.1	......	IRN	.....
Vγ2-S2.9	.....	SWVG	SG......
Vγ2-S1.8	......	Y	AWI.Y.
Vγ2-S2.10	......	MS	AWI.Y.
Vγ2-S6.4	......	ITPRV	.Y.
Vγ2-S1.9	...	SRVVL	YSAWI.Y.
Vγ2-S1.2	.....	L	G......
Vγ2-S1.6	......	S	WI.Y.
Vγ2-S1.14	......	R	YSAWI.Y.
Vγ2-S6.7	......	IGGGS	DV.K.

Vγ3

	Vγ	N region	Jγ
Vγ3-6439	CAYWYK	PRRL	YYYKVF
Vγ3-B2.6	.....	KQ	SG......
Vγ3-B2.1	......	RSAWI	Y.
Vγ3-B2.5	.....	NYR	AWI.Y.
Vγ3-B2.8	....	R	G......
Vγ3-B2.3	......Q	H	DE.I.
Vγ3-B2.2	.....YQG	WSE	......
Vγ3-B2.7	.....YQG	R	......
Vγ3-B1.14	.....YQ	LT	WI.Y.
Vγ3-B1.16	.....YQG	IYGD	.....
Vγ3-B1.13	.....	EV	.....
Vγ3-B2.4	.....	EQGFPT	G......
Vγ3-S6.2	......Q	D	YSAWI.Y.
Vγ3-S1.13	......QG	LR	......
Vγ3-S6.3	......QG	SPSY	GDE.I.
Vγ3-S2.8	......Q	H	DE.I.
Vγ3-S2.10	.....	AR	......
Vγ3-S6.1	.....	NPLE	AWI.Y.
Vγ3-S3.3	.....	EP	WI.Y.
Vγ3-S3.5	.....RQ	AYI	.
Vγ3-S3.6	.....RQ	AR	.
Vγ3-S6.4	.....RQ		E.I.
Vγ3-S2.12	.....RQ	ALSD	DE.I.

Figure 3-4. Predicted amino acid sequences in one letter code of rearranged TCR-γ genes from Brown line and Smyth line chickens, from the conserved Cys94 and Phe108 residues in Vγ and Jγ, respectively. Dots indicate identity to the top line of sequence.

and repertoire selection, and usually express an activated phenotype (Haas et al., 1993; Sciammas et al., 1991). The presence of γδ T cells in the populations of lymphocytes infiltrating tissues affected in various autoimmune diseases suggested a possible role for this T cell subset in the pathogenesis of autoimmunity. which has been further supported by evidence of clonal expansion of the infiltrating γδ T cells in an antigen-driven fashion by findings of related or identical TCR junction sequences (Yurovsky et al., 1994; Yurovsky, 1995; Wucherpfennig et al., 1992; Olive et al., 1992; Shimonkevitz et al., 1993). However, the appearance of γδ T cells after αβ T cells in some autoimmune diseases, such as multiple sclerosis, suggests that they may be recruited secondarily to contribute to the inflammatory response (Zhang et al., 1992). The peripheral blood γδ T cell pool is also expanded in autoimmune diseases, often in a clonal fashion, such as systemic sclerosis (Yurovsky et al., 1994; Yurovsky, 1995), inflammatory bowel disease Bucht et al., 1995), multiple sclerosis (Stinissen et al., 1995), systemic lupus erythematosus (Olive et al., 1994), and insulin-dependent diabetes (Lang et al., 1991). It is unclear whether the expanded peripheral blood γδ T cells are activated in the periphery and then migrate into the affected tissue, or represent spill-over from the inflammatory response in the affected tissue into the periphery. Increased numbers of peripheral blood γδ T cells might also arise from nonspecific polyclonal expansion due to the inflammatory process.

In the avian model of vitiligo, expansion of the peripheral blood γδ T cell population is detectable at 13-18 weeks of age, several weeks after onset of disease at 6-8 weeks, suggesting that their appearance is a secondary event. Human vitiligo patients

with the nonsegmental form of vitiligo have been studied for changes in their peripheral blood lymphocyte subsets by FACS analysis using a panel of mAbs recognizing T cell surface markers (Abdel-Nasser et al., 1992). No significant differences were observed in T cells positive for αβ TCR, γδ TCR, CD3, CD4, CD8, CD45RO, CD11b, CD11c, CD16, CD56, CD25, or CD54. The only changes that were detected included a decrease in the $CD45RA^+$ subset and an increase in $HLA\text{-}DR^+$ cells, suggesting an increase in activated peripheral T cells. Whether this observation is unique to this subset of human vitiligo patients or represents a difference in the pathogenesis or progression of vitiligo in the SL chicken animal model is unknown.

In this report we addressed the issue of clonality of the peripheral blood γδ T cells in the avian model of vitiligo, and found no evidence for clonal expansion of γδ T cells belonging to any of the three subgroups of γδ T cells that can be identified based on Vγ family gene expression. This conclusion is based on analysis of CDR3 length, CDR3 amino acid content and Vγ-Jγ gene combinations expressed in SL peripheral blood as compared to the control parental BL chicken. It should be noted that the number of γδ T cells in the affected tissue, i.e. growing feathers, does increase ten-fold in SL chickens, however, due to the even greater expansion of αβ T cells, the proportion of γδ T cells in the infiltrating T cell population actually is lower than in the feather pulp of normal animals (Erf et al., 1995b). It is unknown whether the infiltrating γδ T cells are clonally expanded.

Future studies might include the use of spectratyping of CDR3 lengths to determine which Vβ and Vγ subfamilies show recurrent usage based on the size of the

CDR3 peaks (Dietrich et al., 1994). This experimental approach might also indicate whether there is clonality within the complex polyclonal repertoire. Single strand conformational polymorphism (SSCP) analysis of amplified PCR products on a nondenaturing polyacrylamide gel would also afford extra characterization of the predominance of certain clones. Diffuse bands and smearing would be indicative of polyclonality; monoclonality or oligoclonality would be confirmed by one or several prominent bands correlating to bulk sequencing.

The developing feather is the tissue of choice to examine the amelanotic process at the site of T cell infiltration. We anticipate that expression of a more restricted T cell repertoire might be found in the regenerating feather, compared with the repertoire found in the T cells of the peripheral blood lymphocytes of the SL chicken. This has been reported for the T cell usage in other autoimmune diseases as well as transplant rejection and tumor infiltrations. For example, an oligoclonal TCR repertoire consisting primarily of Vβ8.2 and Vα2 or Vα4 has been reported for the mouse (Acha et al., 1988; Urban et al., 1988) and rats (Burns et al., 1989) models of EAE. Comparison of the TCR transcripts of T cells found in patient bronchiolar lavage to the same patient's PBL revealed oligoclonal preferences for Vδ1, Vα and Vβ gene families which persisted over time and from multiple tissues (Yurovsky and White, 1995).

In conclusion, the expanded γδ T cell population of SL chickens appears to represent a polyclonal expansion, with no apparent restriction in junctional diversity or significant changes in CDR3 length or amino acid content, or shifts in Jγ gene utilization. These results suggest that the changes observed in PBL γδ T cells in SL chickens are a

consequence of the disease process rather than a causal factor, and may be a secondary result of the inflammatory response at the sites of melanocyte destruction.

CHAPTER 4

ENDOGENOUS VIRAL LOCI IN THE SMYTH LINE CHICKEN: A MODEL FOR THE AUTOIMMUNE DISEASE VITILIGO

Introduction

Viruses have been implicated in the pathogenesis of many autoimmune diseases. Infections by human cytomegalovirus (HCMV) have shown a specific and highly significant association with systemic lupus erythematosus, which has also been associated with retroviruses and Epstein Barr Virus infections (Rider et al., 1997). After an encephalomyelitis infection that has been enhanced by a cryolesion in Lewis rats, cervical lymph nodes appear to be a source of autoimmune lymphocytes involved in cerebral EAE. Reduction in severity of EAE by lymphadenectomy suggested that the lymph nodes prime T cells to target the infected brain (Phillips et al., 1997). There are several murine models of virus-induced diabetes, including lymphocytic choriomeningitis (LCMV), Coxsackie virus, herpes virus, and encephalomyocarditis virus, which is related to the mouse mammary tumor virus (MMTV) and encodes a MHC class II-dependent superantigen via its N-terminal moiety in its *env* gene (Conrad et al., 1997; Ramsingh et al., 1997).

Several mechanisms have been postulated to explain how viral involvement leads to autoimmunity (Aichele et al., 1996; Nakagawa and Harrison, 1996; Barnaba, 1996).

Viruses are involved in the generation of new epitopes (neoantigens) causing a loss of tolerance (breaking of immune ignorance). Immune responses to viral antigenic determinants may trigger cross-reactive autoimmune reactions to shared determinants of the self-antigens that have been released due to tissue destruction during a host antiviral immune response (molecular mimicry) (Douvas and Sobelman, 1991). Viruses may act as superantigens that activate T cells expressing specific Vβ family genes, for example the *Mls* locus in mice, or they may provide immunosuppression, such as in HIV. Respiratory syncytial virus induces host interferon production to inhibit a proliferative response by human PBMCs to the infection (Preston et al., 1995).

In persistent infections in which the body can not completely remove the virus, such as in autoimmune hepatitis, the infected tissue is destroyed during long-term chronic inflammatory responses to the replicating virus. Thus, the destruction is really due to the persistent cytotoxic T cell response on the target tissue. This destruction inadvertently and continuously releases large quantities of the organ's self-antigens (especially never exposed intracellular antigens) which become presented by professional APCs in lymphoid tissue (Koziel et al., 1992; Cerny et al., 1994). Subsequently, self-reactive B cells and T cells become activated to secrete autoantibodies and maintain the autoimmune response.

During infection, some viral protein products can modulate or counteract host antiviral immune defenses (reviewed by Gooding, 1992; Marrack and Kappler, 1994). Cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), have often been the targets for such viral immune modulation. Shope fibroma virus, a pox virus, produces

a protein that binds to TNF preventing the recognition of TNF, by its receptors and thus preventing the activation of inflammatory responses to remove this virus. Cowpox virus encodes a soluble glycoprotein that has amino acid homology to that of the IL-1 receptor. This product probably competes with cell-bound IL-1 receptors for secreted IL-1, interfering with the activation of IL-1 cytokine-mediated inflammatory responses. The product of the *BCRF1* gene of the Epstein-Barr virus stimulates the conversion of T cells into Th2 helper cells by its structural and functional analogy to IL-10. EBV therefore avoids induction of inflammatory responses controlled by Th1 cell activity.

Viruses produce proteins that may subvert the host in other ways as well. Human adenoviruses produce virus-associated (VA) RNAs. These transcripts can regulate interferon activation of P1 kinase by preventing the phosphorylation of eIF-2 (eucaryotic protein synthesis initiation factor), which would prevent the synthesis of viral proteins (Matthews and Shenk, 1991). VA RNAs therefore facilitate continued growth of the virus in the host cells. IgG antibody-dependent complement-mediated destruction of virus- containing cells is avoided by herpes viruses. The herpes simplex virus induces the infected cell to express HSV-Fc receptor, a heterodimer of glycoproteins E and I that binds to the Fc region of the host's nonspecific nonimmune IgG. This binding prevents complement-mediated lysis of infected cells by blocking access to the cell surface of antiviral antibody or effector cells (Bell et al., 1990). Antibodies to herpes simplex virus and CMV Fc-binding proteins have been detected in patients with rheumatoid arthritis (Tsuchiya et al., 1993; Williams et al., 1992). In rheumatoid arthritis, the HLA-DRβ1 alleles contain the QKRAA amino acid sequence in the peptide-binding region that has

been associated with the autoimmune condition. Enhanced humoral and cellular responses to QKRAA sequences expressed by Epstein-Barr virus have been found in RA patients (La-Cava et al., 1997).

Endogenous retroviruses (*ev*) are germline elements that encode components of a retrovirus, are present in all cells, and are inherited in a Mendelian fashion (reviewed in Rovigatti and Astrin, 1983). Endogenous retroviruses are passed from parent to offspring as stable proviral elements integrated into the germline. By reverse transcription from a viral-encoded RNA-dependent DNA polymerase, the DNA copies of these RNA-based viruses have become integrated into the genome of the host flanked by a 5' and a 3' long terminal repeat (LTR). Three genes vital for viral replication are included: *gag*, a precursor polyprotein; *pol*, a precursor for protease, reverse transcriptase, and endonuclease; and *env*, the glycoprotein for the viral envelope. The LTR contains multiple cis-acting elements involved in gene expression and acts as a promoter.

Many *ev* loci are not transcriptionally active; they have truncated open reading frames because of termination codons, deletions or frameshift mutations. However, some *ev* loci are expressed and may actually produce viral particles. Their structural and sequence similarity to infectious exogenous retroviruses, which have been associated with immune dysfunction and tissue-specific expression, make them candidates for pathogenic roles in autoimmunity. Exogenous oncogenic retroviruses also integrate into the host genome and, in the process, cause insertional mutagenesis. In addition, they are not passed from parent to offspring and contain transforming genes such as *v-myc,v-src, v-erb*, and *v-myb,* which are not present in the leukosis viruses or endogenous viruses.

Retroviral integrations may cause immune dysregulation of host cellular gene expression. As transient transposable elements, they can activate or inactivate the host gene by causing gene rearrangements. Retroviruses can undergo gene duplication (of the viral and flanking cellular sequences), capture host genes and move host genetic information by means of intracellular replication and integration as a provirus (Hughes et al., 1981; Tereba, 1981; Rovigatti and Astrin, 1983). The integration of avian leukosis virus into the host's proto-oncogene, *c-myc*, is an example of insertional activation, in this case as a promoter insertion. This brings two coding exons of *c-myc* under the transcriptional control of the 3' LTR and causes higher levels of *c-myc* expression, and the dysregulation of *c-myc* often leads to B cell lymphomas (Coffin, 1991). Other insertional mutations include: (1) enhancer insertions in which the provirus is inserted upstream of the natural or cryptic promoter; (2) leader insertions, which allow readthrough of several transcripts initiated from the provirus 5'LTR; (3) terminator insertions, which install a poly-A signal truncating the transcript, and causing a build up of transcript concentration instead of allowing normal turnover; and (4) insertional inactivation, if the insertion is in the coding region and disrupts function of the gene. As a result of these insertions, the retrovirus genomes often undergo high rates of intragenomic rearrangements such as deletions, point mutations, duplications, and inversions near the 5' LTR in order to relieve a block to transcriptional expression from the 3' LTR Retroviral proviruses can also act as transactivators to the host genome.

Endogenous viruses are ubiquitous in vertebrate species, including humans, and have been extensively studied in White Leghorn lines of the domestic chicken, *Gallus gallus*, where at least 23 endogenous viral *(ev)* loci have been characterized by Southern

blot analysis of junction fragments, inheritance patterns, and structural analysis by restriction mapping and/or DNA sequencing (Humphries et al., 1984; Ronfort et al., 1990). Chicken *ev* loci are structurally related to the avian leukosis and Rous sarcoma virus group (ALV and RSV). They may express *gs* (group specific) or *chf* (chicken factor) antigens, which correspond to the viral *gag* and *env* gene products, respectively, or may produce intact virus (Rovigatti et al., 1983; Smith, 1986). Apparently these endogenous proviruses are not essential; *ev*-negative chickens were found to be normal and fertile (Astrin et al., 1979).

Recent data suggests that human endogenous viruses may be involved in the pathogenesis of a variety of human autoimmune diseases, such as diabetes, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and inflammatory neurologic diseases (Uronovitz and Murphy, 1996). Unique *ev* loci have also been reported in two chicken models for autoimmune diseases. Ziemecki and coworkers (Ziemiecki et al., 1996) described a new locus, designated *ev*22, in the Obese strain (OS), which is characterized by autoimmune thyroiditis, a model for the human organ-specific disease Hashimoto's thyroiditis. Cosegregation was observed of *ev*22 with an OS-specific defect in immunoendocrine communication (a deficient corticosterone response after intravenous injection of lymphokines), but not with T cell hyperproliferation or thyroiditis. Sgonc et al. (1995) reported an association of yet another novel locus, designated *ev*23, in the UCD-200 and UCD-206 chicken lines, which are characterized by hereditary systemic scleroderma-like connective tissue disease. Although *ev*23 may not play a causal role in systemic scleroderma, it is suggested to contribute to disease susceptibility, that is, by prolonging the response of glucocorticoid increasing factors,

such as interleukin-1. Thyroid diseases, type I diabetes, and rheumatoid arthritis, all diseases with autoimmune components, occur with increased frequency in patients with vitiligo (Grimes, 1996).

In this study we sought to determine whether unique *ev* loci are correlated with vitiligo in the SL chicken model by performing Southern blot analysis on genomic DNA from BL and SL chickens. We identified novel *ev* loci not previously described for White Leghorn or other chicken lines, which are segregating in BL and SL chicken lines in a large number of different combinations or *ev* genotypes. Although four *ev* loci, designated *ev*-SL1 through *ev*-SL4, were observed to be present in significantly higher proportions in SL than in BL chickens, none of the *ev*-SL loci was found to be exclusively associated with the vitiligo phenotype when *ev* genotypes for affected and nonaffected SL chickens were compared. However, the genetic polymorphisms detected between BL and SL chickens by these studies suggest that this animal model may be useful for further genetic analysis of vitiligo susceptibility.

Materials and Methods

Southern blot analysis

Genomic DNA samples were prepared from red blood cells of the BL and SL chickens using standard protocols we have previously described (McCormack et al., 1989). Genomic digests were prepared using the restriction endonucleases *Bam*HI and *Eco*RI (New England Biolabs) according to the manufacturer's guidelines, and restriction fragments were separated on 0.8% agarose gels. Southern blots were prepared using Hybond N^+ membranes (Amersham), hybridized at 65°C according to Church and Gilbert

(1984), and washed with 2× SSC, 0.1% SDS at room temperature, followed by 0.1× SSC, 0.1% SDS at 65 °C. The hybridization probe consisted of a ^{32}P-labeled 315 bp *Sac*I-*Eco*RI restriction fragment from the plasmid pU5L (the generous gift of Dr. Maureen Goodenow), representing the U5 region of the avian leukosis virus LTR (Goodenow and Haywood, 1987). This probe hybridizes with both the 5′ and 3′ LTR of avian leukosis virus and endogenous viruses (*ev* loci). Restriction fragments were scored as present or absent. Restriction fragment sizes were calculated based comparison to *Hin*dIII restriction fragments of lambda phage DNA. A single copy probe for the T cell receptor Cβ gene segment (Tjoelker et al., 1990) was used as a control to detect partial digestion.

Statistical analyses

The frequencies of individual *ev* loci, identified as *Bam*HI and *Eco*RI restriction fragments or pairs of fragments, were compared using standard statistical tests, including χ^2 analysis and Fisher's exact test, to determine whether the differences observed between the BL and SL, or between SL progressors and nonprogressors, were statistically significant.

Results

Phenotypic analysis of SL sample population

Thirty-five SL and 13 BL chickens were used for the Southern blot analysis of *ev* loci. Twenty-four of the 35 SL chickens displayed a maximum amelanosis stage of 3-5 according to the amelanosis scale of Erf et al. (1995b) and were classified as progressors in the analyses described below. Eleven SL chickens showed no sign of amelanosis

(stage 1) during their lifetime, and were classified as nonprogressors. The amelanosis stages are indicated in Table 4-1.

Southern blot analysis of BL and SL *ev* loci

Two restriction endonucleases were selected for Southern blot analysis of BL and SL *ev* loci, based on published work describing *ev* loci of other chicken lines. *Bam*HI was selected based on its use to identify characteristic junction fragments for the *ev* loci in White Leghorn chickens (Humphries et al., 1984; Rovigatti and Astrin, 1983), and *Eco*RI was selected based on its use to characterize the *ev* loci of Brown Leghorn chickens (Ronfort et al., 1991). Composites of representative autoradiographs obtained from Southern blots of *Bam*HI and *Eco*RI digests of BL and SL genomic DNA hybridized with the retroviral LTR probe are shown in Figures 4-1 and 4-2, respectively. After hybridization with the LTR probe, all blots were stripped and rehybridized with a single copy Cβ gene probe, which revealed that the restriction digests were complete (data not shown). The frequencies for the presence and absence of *ev* loci, identified as unique *Bam*HI and *Eco*RI restriction fragment or pairs of fragments as discussed below, are summarized in Table 4-2. As discussed below, four *ev* loci of interest are given the designation *ev*-SL.

Within the sample population of BL chickens, 11 of 12 birds exhibited different restriction digest patterns using *Bam*HI, and 12 of 13 birds exhibited different *Eco*RI patterns. A 12 kb *Bam*HI restriction fragment represent the only *ev* locus shared by all BL chickens. The other most common *ev* loci in BL chickens are represented by the 7.3

Table 4-1. Smyth line (SL) chicken phenotypes

Animal #	amelanosis stage*	Animal #	amelanosis stage*
SL 7	4	SL 79	4
SL 10	4	SL 1001	5
SL 16	5	SL 1002	3
SL 22	5	SL 1003	5
SL 23	5	SL 1004	1
SL 26	4	SL 1005	1
SL 28	1	SL 1006	5
SL 31	1	SL 1007	1
SL 34	1	SL 1008	5
SL 35	4	SL 1009	1
SL 44	1	SL 1010	1
SL 55	5	SL 1011	5
SL 61	4	SL 1012	1
SL 64	5	SL 1014	4
SL 65	5	SL 1015	4
SL 70	5	SL 1016	5
SL 72	5	SL 1017	1
SL 77	5		

*The maximum amelanosis stage was classified according to Erf *et al*. (1995a):

1, no amelanosis (nonprogressors)
2, <20% amelanosis
3, 20-60% amelanosis
4, >60% amelanosis
5, complete amelanosis

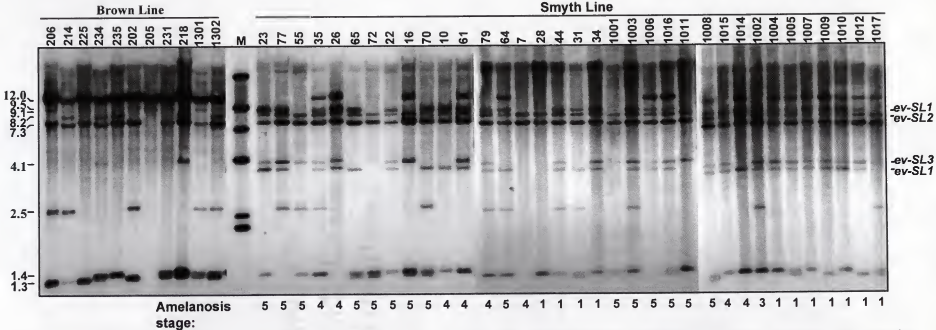

FIG. 4-1. Southern blot analysis of ev loci detected as *Bam*HI restriction fragments. *Bam*HI restriction digests of BL and SL genomic DNA samples were hybridized with an LTR probe. l-*Hin*dIII DNA size markers (lane "M") include 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb (top to bottom). Sizes of bands of interest are indicated on the left, and ev-SL loci are labeled on the right side. Lanes are labeled with individual animal numbers at the top, and the amelanosis stage (Erf et al., 1995a) at the bottom. Southern blots shown are representative of three experiments.

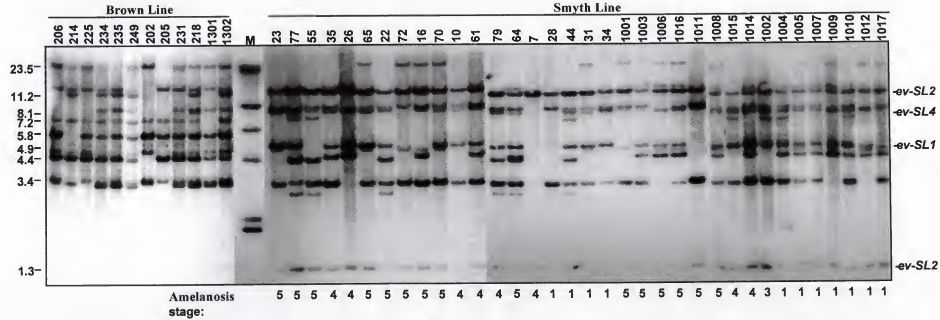

FIG. 4- 2. Southern blot analysis of ev loci detected as *Eco*RI restriction fragments. *Eco*RI restriction digests of Brown line and Smyth line genomic DNA samples were hybridized as described in Figure 4-1. Southern blots shown are representative of three experiments.

TABLE 4-2. Frequencies of *ev* loci detected in BL and SL chickens

Bam HI	BL	BL	SL	SL			*Eco* RI	BL	BL	SL	SL		
band (kb)	present	absent	present	absent	BL *vs*. SL	*ev* loci	band (kb)	present	absent	present	absent	BL *vs*. SL	*ev* loci
9.1 & 3.8	0	12	27	8	p<0.0001	*ev*-SL1	11.2 & 1.3	6	7	35	0	p<0.0001	*ev*-SL2
12	12	0	16	19	p<0.001		4.2 & 2.8	0	13	6	29	n.s.	
9.5	9	3	0	35	p<0.0001		8.4 & 3.2	8	5	29	6	n.s.	
8.2	5	7	35	0	p<0.0001	*ev*-SL2	5.8 & 3.4	12	1	0	35	p<0.0001	
7.3	11	1	35	0	n.s.		23.5	8	5	6	29	p<0.01	
4.1	2	10	26	9	p<0.001	*ev*-SL3	21.7	0	13	4	31	n.s.	
2.5	5	7	11	24	n.s.		12.5	11	2	8	27	p<0.0002	
1.4	7	5	29	6	n.s.		8.1	1	12	32	3	p<0.0001	*ev*-SL4
1.3	8	4	13	22	n.s.		7.2	12	1	7	28	p<0.0001	
							4.9	0	13	27	8	p<0.0001	*ev*-SL1
							4.7	9	4	12	23	n.s.	
							4.4	12	1	19	16	p<0.01	

The table shows the number of chickens in which each *Bam* HI and *Eco* RI band or pair of bands is present and absent (Figs. 4-1 & 4-2).
n.s., not significant; p = Fishers exact test probability value.

kb *Bam*HI restriction fragment (11/12), the 5.8 & 3.4 kb *Eco*RI fragment pair (12/13), and *Eco*RI restriction fragments of 12.5 kb (11/13), 7.2 kb (12/13), and 4.4 kb (12/13).

Within the SL chicken population sample, 27 of 35 chickens exhibited different restriction digest patterns using *Bam*HI, and 26 of 35 birds exhibited different *Eco*RI patterns. Two *ev* loci appear to be shared by all SL chickens. The first of these is represented by the same 7.3 kb *Bam*HI restriction fragment present in 11 of 12 BL chickens. The second *ev* locus present in all SL chickens is represented by the 8.2 kb *Bam*HI restriction fragment and by the 11.2 & 1.3 kb *Eco*RI fragment pair (designated *ev*-SL2, see below). The other most common *ev* locus in SL chickens is represented by an 8.1 kb *Eco*RI restriction fragment present in 32 of 35 birds (designated *ev*-SL4, see below).

Five pairs of restriction fragments are present in the same groups of chickens for a particular restriction digest. Given the large number of different genotypes segregating in the BL and SL populations and the fact that the probe detects both viral LTRs, these restriction fragment pairs are likely to represent the 5′ and 3′ junctions of individual *ev* loci. These restriction fragment pairs include the 9.1 & 3.8 *Bam*HI fragment pairs (*ev*-SL1, Table 4-2), and the 11.2 & 1.3 kb (*ev*-SL2), 4.2 & 2.8 kb, 8.4 & 3.2 kb, and 5.8 & 3.4 kb *Eco*RI fragment pairs (Table 4-2). There were only two exceptions to this pairing of fragments, in that one BL bird (BL214) had a 3.2 kb *Eco*RI band in the absence of a 8.4 kb *Eco*RI band, and one SL bird (SL77) had a 3.4 kb *Eco*RI band in the absence of a 5.8 kb *Eco*RI band.

In three instances there is a perfect correlation between the presence of specific *Bam*HI and *Eco*RI fragments or fragment pairs in the same groups of chickens, suggesting that they represent the same *ev* locus (Table 4-2). These include the 9.1 & 3.8 kb *Bam*HI pair and 4.9 kb *Eco*RI band, representing the *ev*-SL1 locus, the 8.2 kb *Bam*HI band and 11.2 & 1.3 kb *Eco*RI pair representing the *ev*-SL2 locus, and the 1.4 kb *Bam*HI band and 8.4 & 3.2 kb *Eco*RI pair.

The number of *ev* loci per bird for BL and SL chickens is shown in Figure 4-3. *Ev* loci were defined as unique restriction fragment bands or band pairs as described above for each restriction enzyme digest (Hughes et al., 1981). The range for the number of *ev* loci per bird was 2-7 for BL and SL chickens, as detected by Southern blots of *Bam*HI digests, and 1-9 as detected by Southern blots of *Eco*RI digests. The average number of *ev* loci per bird did not differ significantly between the BL and SL groups, with average numbers of 4.9 ± 1.2 for BL (n=12) and 5.5 ± 1.1 for SL (n=35) chickens based on the *Bam*HI Southern blots, and 6.2 ± 1.4 for BL (n=13) and 5.3 ± 1.4 for SL (n=35) chickens based on the *Eco*RI Southern blots.

The BL and SL *Bam*HI restriction fragments do not co-migrate with the White Leghorn loci *ev*1 or *ev*5 (data not shown). The 7.3 kb *Bam*HI fragment present in nearly all BL and SL chickens comigrates with *ev*3 (data not shown), but its identity to *ev*3 is ruled out by the absence of *Eco*RI fragments of the appropriate size (Hughes et al., 1981). Although standard markers for all known *ev* loci have not been compared to the *ev*-SL loci by Southern blot analysis, comparison of the restriction fragment sizes summarized in Table 4-2 with published data for *ev* loci already reported for White Leghorns (Humphries et al., 1984; Rovigatti and Astrin, 1983), Brown Leghorns (Ronfort et al.,

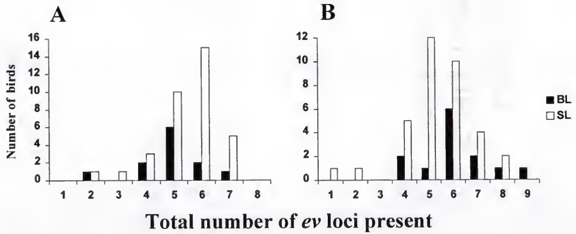

FIG. 4-3. BL and SL chickens have similar total numbers of ev loci. The numbers of ev loci detected in individual BL and SL chickens by (A) *Bam*HI and (B) *Eco*RI restriction digests are plotted against the number of birds with each number of loci. Filled bars, BL; open bars, SL.

1991) and broilers (Boilliou et al., 1991) suggests that BL and SL chickens are characterized by unique *ev* loci. In particular, none of the restriction fragments representing *ev*-SL loci of interest, *i.e.* *ev*-SL1 through *ev*-SL4, correspond in size with those of previously identified *ev* loci. Taken together, these data suggest that a large number of previously undescribed *ev* loci are segregating in BL and SL chicken.

Comparison of BL and SL *ev* genotypes

Statistical analyses of the frequencies for the presence and absence of *ev* loci in BL and SL chickens are shown in Table 4-2 for the *Bam*HI and *Eco*RI restriction digests. Four *ev* loci, which we have designated as *ev*-SL1 through *ev*-SL4, were observed to be present in significantly higher proportions in SL than in BL chickens ($p<0.001$ by Fishers exact test). The *ev*-SL1 and *ev*-SL2 loci are identified on both the *Bam*HI and the *Eco*RI Southern blots, whereas *ev*-SL3 and *ev*-SL4 are identified on *Bam*HI or *Eco*RI blots, respectively (Figures 4-1 and 4-2; Table 4-2). The number of *ev*-SL loci detected per bird and the *ev*-SL locus combinations observed are shown in Figure 4-4. Most SL chickens (32/35) have 3 or more *ev*-SL loci, whereas most BL chickens (10/12) have one or none. As already noted above, *ev*-SL2 is present in 100% (35/35) of the SL chickens tested, but is present in only 38% (5/13) of the BL chickens. The *ev*-SL1 locus is absent from the BL sample population, but present in 77% (27/35) of the SL chickens tested. The *ev*-SL3 and *ev*-SL4 loci were present in 74% (26/35) and 91% (32/35) of SL chickens, respectively, and in only 17% (2/12) and 8% (1/13) of BL chickens, respectively. As shown in Figure 4-4, the only *ev*-SL combinations observed in either the BL or SL were *ev*-SL2+3, *ev*-SL1+2+4, *ev*-SL2+3+4, and all four *ev*-SL loci.

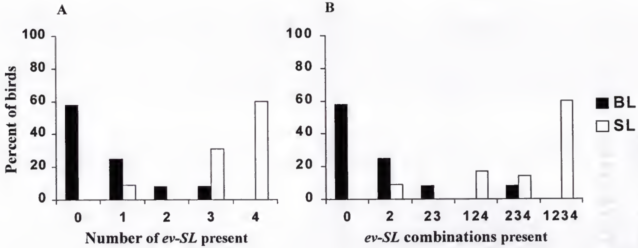

FIG. 4-4. SL chickens have more *ev*-SL loci than BL chickens. (A) The numbers of *ev*-SL loci detected in individual BL and SL chickens are plotted against the percent of birds with each number of loci. (B) The *ev*-SL genotypes present are plotted against the percent of birds with each genotype. Filled bars, BL; open bars, SL.

Other significant differences between BL and SL chickens were observed that correlated with the relative absence of some *ev* loci in SL chickens. As many as seven *ev* loci, which we have not assigned any designations pending further characterization, were observed to be present in significantly higher proportions in BL than in SL chickens ($p<0.01$ by Fishers exact test). The *ev* loci that are present in significantly higher proportions of BL than SL chickens include the 12 and 9.5 kb *Bam*HI fragments, and the 5.8 & 3.4 kb *Eco*RI fragment pair and the 23.5, 12.5, 7.2, and 4.4 kb *Eco*RI fragments (Table 4-2). These data suggest that *ev* loci may be useful as genetic markers to distinguish between BL and SL chickens and/or other chicken lines.

Comparison of SL progressor and SL nonprogressor *ev* genotypes

The *ev* genotypes were compared for SL subpopulations defined on the basis of their maximum stage of amelanosis, in order to determine whether there was any correlation between the presence of specific *ev* loci with the vitiligo phenotype. No significant differences in the frequency of individual *ev* locus presence could be associated with the SL progressor (stages 2-5) phenotype, as compared to the SL nonprogressor (stage 1) phenotype (Table 4-3). The number of *ev*-SL loci detected per bird and the *ev*-SL locus combinations present were similar in SL progressors and nonprogressors. These data suggest that, in contrast to two other chicken models of autoimmunity (Sgonc et al., 1995; Ziemiecki et al., 1988), there is no unique *ev* locus associated with vitiligo in the SL chicken.

Table 4-3. Frequencies of *ev* loci detected in SL progressing (p) and nonprogressing (np) chickens

Bam HI band (kb)	SLp present	SLp absent	SLnp present	SLnp absent	SL p vs. np	*ev* loci	*Eco* RI band (kb)	SLp present	SLp absent	SLnp present	SLnp absent	SL p vs. np	*ev* loci
9.1 & 3.8	18	6	9	2	n.s.	*ev*-SL1	11.2 & 1.3	24	0	11	0	n.s.	*ev*-SL2
12	9	15	7	4	n.s.		4.2 & 2.8	5	19	1	10	n.s.	
9.5	0	24	0	11	n.s.		8.4 & 3.2	21	3	8	3	n.s.	
8.2	24	0	11	0	n.s.	*ev*-SL2	5.8 & 3.4	0	24	0	11	n.s.	
7.3	24	0	11	0	n.s.		23.5	3	21	3	8	n.s.	
4.1	17	7	9	2	n.s.	*ev*-SL3	21.7	4	20	0	11	n.s.	
2.5	8	16	3	8	n.s.		12.5	7	17	1	10	n.s.	
1.4	20	4	9	2	n.s.		8.1	23	1	9	2	n.s.	*ev*-SL4
1.3	8	16	5	6	n.s.		7.2	5	19	2	9	n.s.	
							4.9	19	5	8	3	n.s.	*ev*-SL1
							4.7	8	16	4	7	n.s.	
							4.4	13	11	6	5	n.s.	

The table shows the number of chickens in which each *Bam* HI and *Eco* RI band or pair of bands is present and absent (Figs. 4-1 & 4-2).
n.s., not significant; p = Fishers exact test probability value.

Discussion

The inheritance of vitiligo in the Smyth line animal model is polygenic in nature, and shows low penetrance in out-cross matings (Smyth et al., 1981). The incidence and severity of the amelanosis and other associated autoimmune defects (hypothyroidism and alopecia-like feathering defect) in the SL chicken are influenced by a variety of factors, both genetic and environmental. Although the major histocompatibility complex (MHC) is an important genetic factor, as evidenced by differences in disease severity and age of onset associated with different MHC alleles in SL sublines (Erf et al., 1995a), the BL and SL chickens used in this study were MHC-matched, thus ruling out MHC influence. Other genetic factors appear to include inherent melanocyte defects, melanin pigmentation genes, melanocyte-stimulating hormone, and sex hormones (reviewed in Smyth, 1989). The varying incidence of amelanosis in different SL breeding colonies from 60-90% suggests that some environmental factors may be associated with housing conditions (McCormack and Smyth, unpublished).

Human vitiligo also appears to be polygenic, and is suggested to result from recessive alleles at several unlinked autosomal loci based on extensive familial aggregation of vitiligo (Bhatia et al., 1992; Majumder et al., 1993), and the observation that 20% of probands are reported to have at least one first-degree relative affected by vitiligo (Nath et al., 1994). It has been speculated that simultaneous alterations in several genes are required to cause disease or increase susceptibility, e.g. mutations in genes controlling melanocyte growth and survival and/or the immune response (Lacour and

Ortonne, 1995). Candidate genes for vitiligo susceptibility might be suggested by defects in enzymes involved in melanogenesis and catecholamine metabolism that have been associated with vitiligo (Halaban and Moellmann, 1993; Austin and Boissy, 1995; Salzer and Schallreuter, 1995; Schallreuter et al., 1994). Although there are some reports that specific HLA class I or II alleles are associated with vitiligo in some human populations (Dunston and Hadler, 1990; Vennecker et al., 1992; Ando et al., 1993; al Fouzan et al., 1995), other investigators report no such association (Schallreuter et al., 1993; Huang et al., 1996).

In this study we have taken advantage of the genetics of endogenous viruses in chickens to explore the possibility that endogenous viruses may play a role in genetic susceptibility to vitiligo in the avian model. Nearly all chickens have *ev* loci, which may be silent or expressed as viral proteins or infectious virus, and *ev* gene expression can influence the course of infection by exogenous avian leukosis virus (Rovigatti and Astrin, 1983). There are reported associations between various specific *ev* loci and commercially important production traits in chickens (Govora et al., 1991; Iraqi et al., 1994), however, it was the two reports of association of novel *ev* loci with two other autoimmune diseases that prompted us to examine *ev* loci in SL chickens, including the OS chicken with spontaneous hereditary thyroiditis (Ziemiecki et al., 1988) and the UCD-200 and 206 lines with hereditary systemic scleroderma-like connective tissue disease (Sgonc et al., 1995).

There are several possible roles of *ev* loci in susceptibility to autoimmune diseases. One possibility is that one or more *ev* loci disrupts a gene expressed in the target tissue, contributing to an increased susceptibility to autoimmune recognition, or in

lymphocytes, resulting in a possible breakdown of normal self-tolerance. An example of such an insertional mutagenesis event in an autoimmune disease was reported by Wu and colleagues (1993), who observed that integration of an endogenous retrovirus has occurred in the Fas apoptosis-regulating gene of MRL-*lpr/lpr* mice. Alternatively, *ev* loci may represent genetic markers for linked susceptibility genes. A third possibility is that one or more *ev* loci produces infectious viral particles, resulting in a somatic reinfection of cells involved in the autoimmune process as suggested above. Whether human endogenous viruses are involved in vitiligo remains to be determined, although their possible involvement in human neoplastic and autoimmune disease has been suggested (Hohenadl et al., 1996; Urnovitz and Murphy, 1996).

We identified four *ev* loci that are present at statistically significantly higher frequencies in SL than BL chickens, and an additional seven *ev* loci characteristic of BL chickens. The predominance of the *ev*-SL loci may have originated during the original selection of SL chickens and/or may have been introduced during derivation of the SL from outcrosses with other chicken lines (Smyth et al., 1981). When the *ev* genotypes for SL progressors and nonprogressors were compared, there was no significant association of *ev* loci with the autoimmune phenotype, thus, in contrast to the OS and UCD-200 avian models of other human autoimmune diseases, we find no evidence for a role of *ev* loci in the pathogenesis of vitiligo in the SL chicken animal model.

Possible roles for endogenous viruses in the pathogenesis of avian amelanosis might be detectable with additional experiments. DNA methylation has been suggested as being partially responsible for the low expression of some avian endogenous viruses. An inhibitor of DNA methylation, 5-azacytidine can induce the expression of viral

particles in *ev*-1 containing chicken embryo cells (Rovigatti and Astrin, 1983). 5-azacytidine treatment activates silent genes and promotes cellular differentiation. 5-azacytidine has been shown to increase the incidence of autoimmune thyroiditis in the susceptible parental line (Cornell C strain) of the Obese strain chicken (Schauenstein et al., 1991). Chronic low dose administration of 5-azacytidine induced amelanosis in the genetically susceptible Brown Line chickens, but not the more distantly related LBL chickens (Sreekumar et al., 1996). However, the effects of 5-azacytidine treatment on *ev* gene expression in BL and SL chickens have not been reported.

If experimental evidence is found to implicate a particular *ev* locus in the pathogenesis of vitiligo, it could be identified in a genomic library using appropriate probes and restriction mapping, cloned and then sequenced in order to determine homology with that of known retroviruses. In situ hybridization of the specific *ev* sequence can be used as a probe to metaphase chromosomes to localize the *ev* loci. Six *ev* loci in chickens have be mapped to chromosome 1 alone (Tereba et al., 1979; Tereba and Astrin, 1980). In addition, the phenotype of the *ev* locus would be characterized for the presence of the *gs* antigen (indirect immunoflourescence), of the *chf* (fusion with the 16Q cell line), detection of the virus (which subgroup) or viral proteins from the culture supernatant, and the susceptibility to other subgroups of the ALV-RSV group (Humphries et al., 1984a). Endogenous retroviruses are in subgroup E and are nononcogenic, unlike the ALVs of the exogenous viruses found in subgroups A, B, C, and D. Expression of subgroup E viral proteins helps make the cells refractory to infection by other viruses of the same subgroup. Cells containing *ev*3, *ev*6, and/or *ev*9

have a reduced susceptibility to infection to exogenous subgroup E virus (Rovigatti and Astrin, 1983; Robinson et al., 1981).

The heterogeneity of the endogenous viral loci detected in this study for the SL and BL chickens is interesting considering the fact that the SL was derived from the BL. This may be due to the outcrossings used to establish the SL (Smyth et al., 1981) *i.e.*, the outcrossing to Barred Plymouth Rock, Light Brown Leghorns, and a random-breeding meat stock may have introduced additional *ev* loci.

An alternative approach to genetic mapping of vitiligo susceptibility is genome-wide genetic linkage analysis. Sets of primer pairs for microsatellite genetic markers are now available from the U.S. Poultry Gene Mapping Project. Mapping backcross progeny with these markers will assist in the identification of candidate regions associated with vitiligo. Candidate genes may ultimately be identified within these genomic intervals, which can then be analyzed for gene expression, identity of the protein, mutational analysis and functional assays to determine the cause of amelanosis.

One approach to examine gene expression would make use of 5-azacytidine treated BL chickens. Northern blot analysis could be used to compare gene expression in SL, BL, 5-azacytidine-treated BL, and LBL chickens. One might predict differences in expression of candidate genes when comparing the 5-azacytidine-treated BL and SL to untreated BL and LBL chickens.

Interestingly, of the approximately 100 BL chickens we have raised, we have recently identified one BL chicken expressing amelanosis (stage 4). The *ev* genotype of this amelanotic BL chicken resembled a SL genotype, i.e. all four *ev*-SL loci were present and several *ev* loci characteristic of BL chickens were absent (data not shown),

suggesting that the genome of this individual BL chicken is more similar to SL chickens than to other BL chickens. This observation is consistent with the hypothesis that the 1-2% incidence of amelanosis observed in BL chickens (Smyth et al., 1981) is due to the chance combinations of multiple recessive vitiligo susceptibility genes segregating in the Brown line, and that these genes were selected for during the derivation of the Smyth line. In conclusion, although we find no evidence of linkage of *ev* loci with the vitiligo phenotype in SL chickens, our results suggest that BL and SL chickens bear considerable genetic polymorphisms, and will therefore provide a useful model for further genetic dissection of vitiligo susceptibility.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

As autoimmune diseases are explored many have witnessed that finding a simple answer is doubtful. The process of amelanosis found in the Smyth line chicken is another example of a complex situation. There is obvious evidence that it is a polygenic phenomenon. Three sublines of the Smyth line chicken exist as the B^{101}, B^{102}, and the B^{103}. The B^{101} subline has the quickest onset and greatest severity but all three had a high incidence of amelanosis between 80% and 90%. Yet even in the B^{101}, there is variability. Females usually display a quicker onset, pass through the stages more rapidly, and achieve the higher stages of amelanosis than the males. However, not all females will develop amelanosis. Some will never become amelanotic, while others do not even begin depigmentation until the 17th week of life and do not progress to the severe stages. In the SL2 population, three females reverted and became partially repigmented after they became sexually mature. Most of the male B^{101} never became amelanotic. Of the male B^{101} chickens that did become amelanotic a few became just as severe as the females with similar ages in terms of onset and progression. Some and only female SL chickens were blind, as noticed by their unawareness when they were being picked up. There were females that displayed alopecia, the feathering defect.

Thus the variability of amelanosis in the Smyth line B subline characterizes the polygenic variability in the rate, penetrance and environment. Smyth reported that in

Massachusetts, 90% of the SL developed amelanosis. In comparison, the UF SL colony is characterized by a 60% incidence of amelanosis while the colony of Dr. G. Erf (University of Arkansas), which is raised under specific pathogen-free conditions, has an even lower incidence of amelanosis (personal communication). The Brown line chickens of the same MHC haplotype, B^{101}, have a susceptibility of 1-2%. One BL, BL7-1130, did spontaneously become amelanotic at the same early onset and severity of condition as the typical SL we've studied. The BL hosts in the adoptive transfer experiments that demonstrated amelanosis might indeed have had the genetic combination to be more susceptible to develop amelanosis on their own, as demonstrated in the BL7-1130. Perhaps the transferred cells helped to potentiate and quicken what was already there. This would reflect the work of Wicker's lab. They felt that the transfer of splenocytes from affected NOD mice into young NOD females yielded the quicker onset and severity to already susceptible genetics within the hosts (Wicker et al., 1986).

The fact that the splenocytes were able to transfer susceptibility to the BL hosts is an indication that T cells mediate some aspect of amelanosis. As cited before, the works of Austin et al. (1992, 1995), Boissy et al. (1985) and Lamont et al. (1981, 1982) have proven a role of autoantibodies by bursectomy, which basically eliminates the B cell population in chickens. The anti-melanocyte autoantibodies detected melanocyte proteins of 65-80kDa molecular weights, and the latest evidence shows that these represent binding to the possible autoantigen Trp-1. The drug cyclosporin, an inhibitor of all T cells has been shown to suppress amelanosis in the SL chicken (Pardue, 1987). The experiments described herein, provide the first *in vivo* evidence that T cells can at least

transfer a potentiation of the amelanotic disease susceptibility found in the Smyth chicken.

It would thus be worth expanding these T cell transfer experiments. As stated earlier, fewer restrictions to the experiment need to be in place to better assure its success. I would suggest an experiment patterned using a series of repeated donations of affected SL splenocytes, using cyclophosphamide to provide the immunosuppression. Cyclophosphamide does not affect T cells and untreated chicks are reported to have a better survival rate (Toivanen et al., 1975; Lehtonen et al., 1990). A dose dependence curve should be generated to optimize the quality of immunosuppression despite the sacrifice of an initial set of birds. A more pathogen-limited environment in which to raise the immunocompromised hosts during the initial recovery from immunosuppression is suggested. The use of 5-azacytidine may help to potentiate the manifestation of the amelanotic phenotype of the hosts as seen in the work by Sreekumar and colleagues (1995); but it might disguise the cell transfer. Mitogen-stimulation such as by Con-A of the donor splenocytes may also help to potentiate the observation of amelanosis in the transfer hosts.

If repeatable results continue, then T cell subsets may further help define the involved autoimmune T cells. Separation of chicken T cell subsets is made possible with available reagents that include $\gamma\delta$ T cells, $\alpha\beta$ T cells expressing $V\beta1$ genes, and $\alpha\beta$ T cells expressing $V\beta2$ genes, which are identified by the mouse monoclonal antibodies, TCR1, TCR2, and TCR3, respectively.

A TCR repertoire analysis of the regenerating feather may characterize the expansion that appears to be indicated in the intense lymphocytic infiltration of the pulp as reported in the work of Erf et al. (1995a, 1995b). Counts of cells in cryosections stained with mouse anti-TCR antibodies have indicated high presence for both $CD4^+$ and $CD8^+$ T cells expressing predominantly $\alpha\beta$ TCR bearing Vβ1 (i.e. $TCR2^+$). So the repertoire analysis should still be initially examining Vβ1. If an oligoclonal expansion of certain recurring subset(s) of T cells is detected from the feather analysis, then it might be possible to isolate a subset of T cells from regenerating feathers, which could be cultured, stimulated with Con-A, and then transferred into 5-6 week BL hosts. This population might be more reflective of the autoantigen-activated cells than splenic cells.

More importantly, this would lead to possible immunotherapy. Monoclonal antibodies directed against the specific Vβ chain (for example) might be used to inactivate that autoreactive subset of T cells while leaving the rest of the T cell repertoire intact. This Vβ selective therapy has been performed experimentally in the treatment of chronic relapsing EAE in SJL/J mice by Whitham and colleagues (1996). This would bridge the work between the TCR repertoire analysis and the adoptive transfer studies.

By detecting endogenous virus integrations as inheritable stable elements in the genomes of the Smyth line chicken, four novel loci (*ev*-SL) have been identified that may be used as genetic markers for vitiligo susceptibility in this chicken. Although these loci were not unique to only the SL birds displaying the amelanotic phenotype, it may still be possible to find loci that are correlated with the disease. Associations of novel *ev*-loci have been found in the two other major chicken models for autoimmunity, the OS

chicken for thyroiditis and the UDC-200 and UCD-206 chickens for systemic scleroderma.

An improvement in possibly detecting vitiligo susceptibility genes is to use a genome-wide linkage analysis, mapping backcross progeny between the SL and BL chickens. Using primer pairs for microsatellite genetic markers from the U.S. Poultry Gene Mapping Project, I would like to identify candidate genetic intervals that are associated with vitiligo. This would assist in the identification of vitiligo-associated candidate regions. The candidate genes may be identified from these candidate regions by physical mapping. Yac constructs would be created and linked into contigs. Within each Yac construct, a more dense use of microsatellite markers would be used to further narrow and fine map the intervals within a candidate region. A candidate gene could be identified in a genomic library and then be confirmed by in situ hybridization to chromosome spreads using the sequence of a cloned restriction fragment from the candidate locus.

Once a gene is cloned, then the more interesting questions can be examined. I will need to translate its protein sequence and look for its identity by a search for homology to known proteins in GenBank or similar database. If the protein is well characterized, such as IL-4, then functional assays of IL-4 and the pathogenesis of vitiligo could be examined. Mutational analysis by introducing a transgene containing a reporter gene such as the *lac* gene or a gentimycin resistance gene could be introduced in the chick embryo to create a knockout and see if vitiligo is recreated in a BL. The effects of over- or under-expression of the protein can be assessed.

What has still not been addressed is the inherently defective melanocyte itself. Still available are original plans to perform antibody dependent cell cytotoxicity assays. These would consist of incubating cultured SL or BL melanocytes (all of the B^{101} haplotype) from cultures with either affected or nonaffected SL sera or BL sera and either White Leghorn complement or White Leghorn spleen cells as effectors of cytotoxicity. This will examine the surface expression of the SL melanocyte and detect aberrant expression of melanocyte autoantigens as compared to that expressed by BL. Such autoantigens might be those involved in melanin production, including TRP-1. If the sera of the SL can activate the destruction of both BL and SL melanocytes or if the sera of the BL can not activate the destruction of either of the melanocytes, then the melanocyte is not different between the two strains, at least at the level of surface expression of key autoantigens.

Experiments detecting differences in RNA and protein expression between SL and BL melanocytes and T lymphocytes are another direction. This would require that a library of primers for chicken cytokines be readily available, unless one is willing to optimize the conditions for using mammalian primers. Cultures would include T cells, melanocytes and APCs. PCR amplification from mRNA isolated from the cultures would allow the detection of the types of cytokines released by the T cells. The identification of IL receptors may be detected as well.

A cDNA library of the mRNA produced by cultured melanocytes could be established. From the survey conducted, identity of candidate vitiligo susceptible loci can be determined. The cDNA would be cloned into vectors and the DNA of candidate vitiligo susceptible loci sequenced. The sequence could be translated into an amino acid

sequence and compared with sequences submitted in GenBank or similar database. This may characterize and reveal the possible aberrant nature of the SL melanocyte.

The nature of the proteins expressed may reveal possible epitope differences between the SL and BL melanocytes. It may confirm or corroborate with the work of Boissy and colleagues in the identification of autoantigens (Austin et al., 1992; Austin and Boissy, 1995). It may lead to oral immunotherapy experiments using peptide administrations similar to the administrations of MBP into EAE mice and GAD into NOD mice to induce tolerance.

Thus, this was my study of amelanosis in the Smyth line chicken as an animal model for the autoimmune disease of human vitiligo. Vitiligo is just one piece in the autoimmune puzzle. As one of many autoimmune diseases, it is my hope, as well as others, that a common factor or theme of factors can be found and that I would in some way have helped improve the lives of many.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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